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THE EFFECT OF INCUBATION TEMPERATURE ON THE RECOVERY OF SPORES OF *BACILLUS SUBTILIS* 8057

by

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ABSTRACT

A temperature gradient incubator has been built which allows replicate colony counts to be carried out in roll tubes over a wide range of incubation temperatures. This incubator was used to study the recovery of B. subtilis spores in Plate Count Agar (PCA) and Brain Heart Infusion Agar after heat-treatment at 95° for different times and subsequent incubation at different temperatures. With both media, unheated spores showed similar recoveries in the range from 16 to 48° whereas severely heated spores gave optimum recovery in the region of 30°. Treatment at 105° showed a similar trend during recovery on PCA.

A study of the effect of incubation temperature on the rate of germination as shown by loss of optical density showed that untreated spores gave a maximum germination rate at c. 41° and ceased to germinate at c. 50°. Spores heated for 20 min at 95° could germinate up to 52.5°, a temperature which allowed no recovery. This suggests that the recovery of heat-treated spores at different temperatures is not limited by their ability to germinate. The rate of germination at 30° as shown by loss of heat-resistance was found to be slower for heat-treated spores than for untreated spores.

Density gradient centrifugation of a spore suspension in both renografin and sucrose gave two distinct bands. The lighter fraction was shown to consist of germinated spores and the heavier fraction of ungerminated spores, thus demonstrating a method of separating germinated from ungerminated spores. This technique was used to obtain germinated

spores from suspensions which had previously been subjected to different heat-treatments at 95°. A study of the effect of different incubation temperatures on the outgrowth of these germinated spores showed that as the heat-treatments became more severe, the temperature range giving maximum outgrowth was reduced in the same fashion as the recovery of ungerminated spores. It was therefore concluded that it is the outgrowth of the germinated spore which causes temperature sensitivity during recovery.

Germinated spores obtained by incomplete germination of a heat-treated suspension showed less temperature sensitivity during outgrowth than those obtained by germination for a longer time, suggesting that the least damaged spores in a suspension are least affected by the temperature of recovery.

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I. INTRODUCTION

A. General

Spores occur in nature as the dormant form of several genera of bacteria, the most commonly occurring being those of *Bacillus* and *Clostridium*. These are widespread and have been shown to be present in such diverse environments as Antarctic soil (Marshall and Ohye, 1966) and marine sediments (Smith, 1968). Another important characteristic of bacterial spores is their high resistance to environmental extremes thus allowing their survival in conditions which would be lethal for many other forms of life.

Because of their ubiquity, microbes from sources such as soil, water, faeces and air are present in foodstuffs and since they are responsible both for spoilage and food poisoning, they must be destroyed or made quiescent before food can be stored for any length of time. Since in food processing heat is the most commonly used method of reducing microbial populations and since many bacterial spores are particularly resistant to heat, any process intended to render a product sterile or nearly so must be capable of killing spores in the raw foodstuff.

Slepecky (1972) has stated, "Of all microbial populations, the sporeformers, because of their heat resistance, have been one of the most important considerations in food processing and the most influential in terms of commercial technology".

B. The Nature of Spores

1. The Formation of Bacterial Spores

The formation of spores from the vegetative cell (sporogenesis) takes place in a series of biochemical and morphological changes which Halvorson (1965) has shown schematically in the following stages (see Fig. 1).

A. This is the irreversible commitment to sporulation. It is accompanied by the production of a sporulation factor, an antibiotic and a protease. The sporulation factor appears at the time of granulation and initiates sporulation (Sussman and Halvorson, 1966).

B. Acetate oxidation involving the TCA and glyoxylic acid cycle occurs here.

C. Ribosidase is formed at this stage.

D. The spore becomes more resistant to irradiation with the formation of S-S proteins.

E. About 2 h after filamentation commences, dipicolinic acid is synthesized and calcium is incorporated in the spore.

F. The spore becomes heat-resistant.

G. Alanine racemase is synthesized in the spore. This heat-resistant enzyme is thought to be associated with the outer spore coat or exosporium and little of it is to be found in the vegetative cells (Stewart and Halvorson, 1953).

H. A lytic system is produced.

I. The spores are released from the parent cell.

Although the genetic factors controlling this sequence of

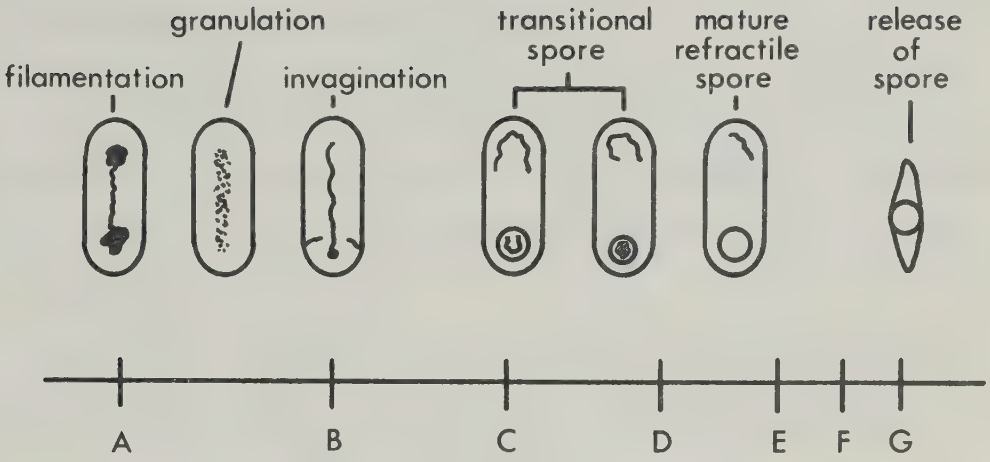

Fig. 1. Sequential appearance of morphological and biochemical components during sporulation in <u>Bacillus</u> species. (Adapted from Halvorson, 1965).

events are very complex and are not fully understood, most workers in this field agree that spore formation is most likely initiated by some form of catabolite derepression.

2. The Structure of the Bacterial Spore

The composition of the bacterial spore has recently been comprehensively reviewed by Murrell (1969) therefore, for the purposes of this thesis only a brief description of the main features shown in Fig. 2 will be given.

Exosporium. The exosporium is thought to be a phospholipoprotein complex similar to that of unit membranes with in addition sugars and glucosamine. Little more information is available.

Coats. The spore coats are responsible for the shape of the cell, 50% of the volume and 40 - 60% of the dry weight. They consist of very stable disulphide rich proteins combined with lipids and phosphorus, suggesting a phospholipoprotein layered structure.

Cortex. This is thought to be a mucopeptide of a structure similar to vegetative cell walls, appearing as a layered matrix probably cross linked. (A hypothetical structure for the cortex, proposed by Murrell and Warth (1965) is given in Fig. 3.)

Germ Cell Wall. This layer has not yet been isolated and analyzed but is thought to be a mucopeptide which is eventually incorporated into the cell wall of the newly formed vegetative cell.

Protoplast. This contains the cytoplasm, nucleus and plasma membrane of the mature spore.

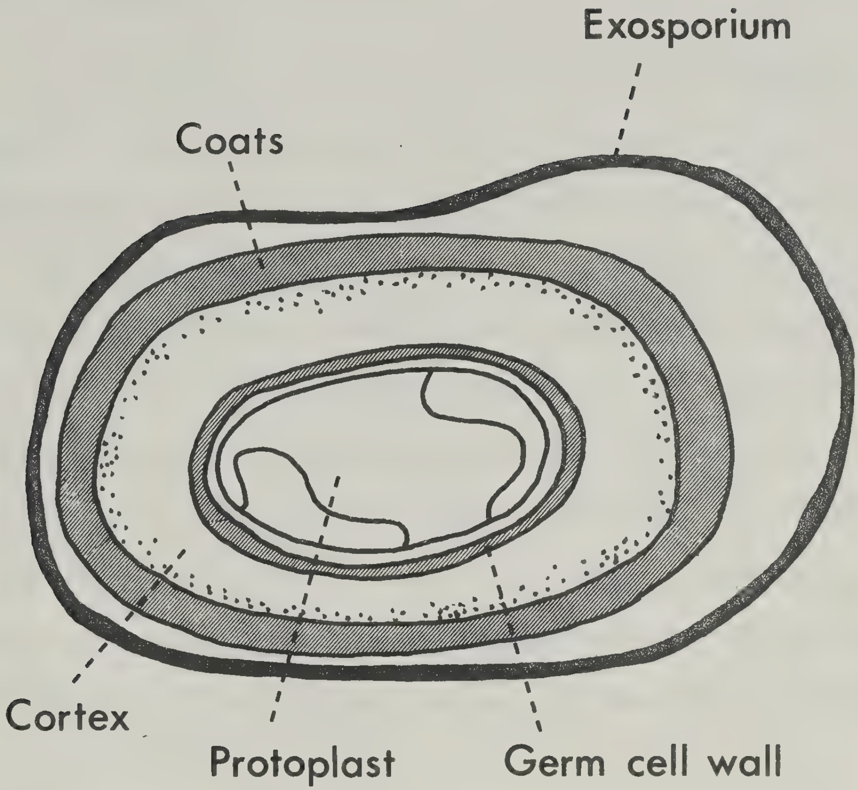

Fig. 2. Diagrammatic representation of the structure of the bacterial spore (Gould and Hurst, 1969).

3. The Resistance of Bacterial Spores

The mechanism of spore resistance is not well understood and seems not to depend on any one factor in the cell. Some of the following factors are thought to be associated with resistance.

a) Spore coat. Spores produced in the presence of chloramphenicol were found to be deficient in coat layers (Ryter and Szulmajster, 1965). Although these spores were refractile and resistant to heating at 80° for 10 min, they were unstable and germinated in a few days.

Knaysi (1938) suggested that the resistance of spores might be related to the toughness of their coats, however Warth *et al*. (1963a) showed the coats of *Bacillus stearothermophilus* spores to be more easily ruptured by grinding than the coats of many less resistant spores.

b) Cortex. Cortex-deficient spores produced in the presence of penicillin were found not to be heat-resistant (Fitz-James, 1963). Penicillin prevents the formation of the cortex mucopeptide polymers and thus, although both Ca^{++} and dipicolinic acid were taken up normally during sporulation, they were released into the medium on terminal lysis of the cell. It thus seems that an intact cortex is necessary for heat-resistance.

Murrell and Warth (1965) in a study of *Bacillus subtilis* spores with a wide range of heat-resistances showed that there was a relationship between the diaminopimelic acid (DAP) content and the heat-resistance of the cell. No such relationship existed between the heat-resistance and the hexosamine content. They postulated a structure for the spore cortex (Fig. 3) and suggested that since it is the $-NH_2$

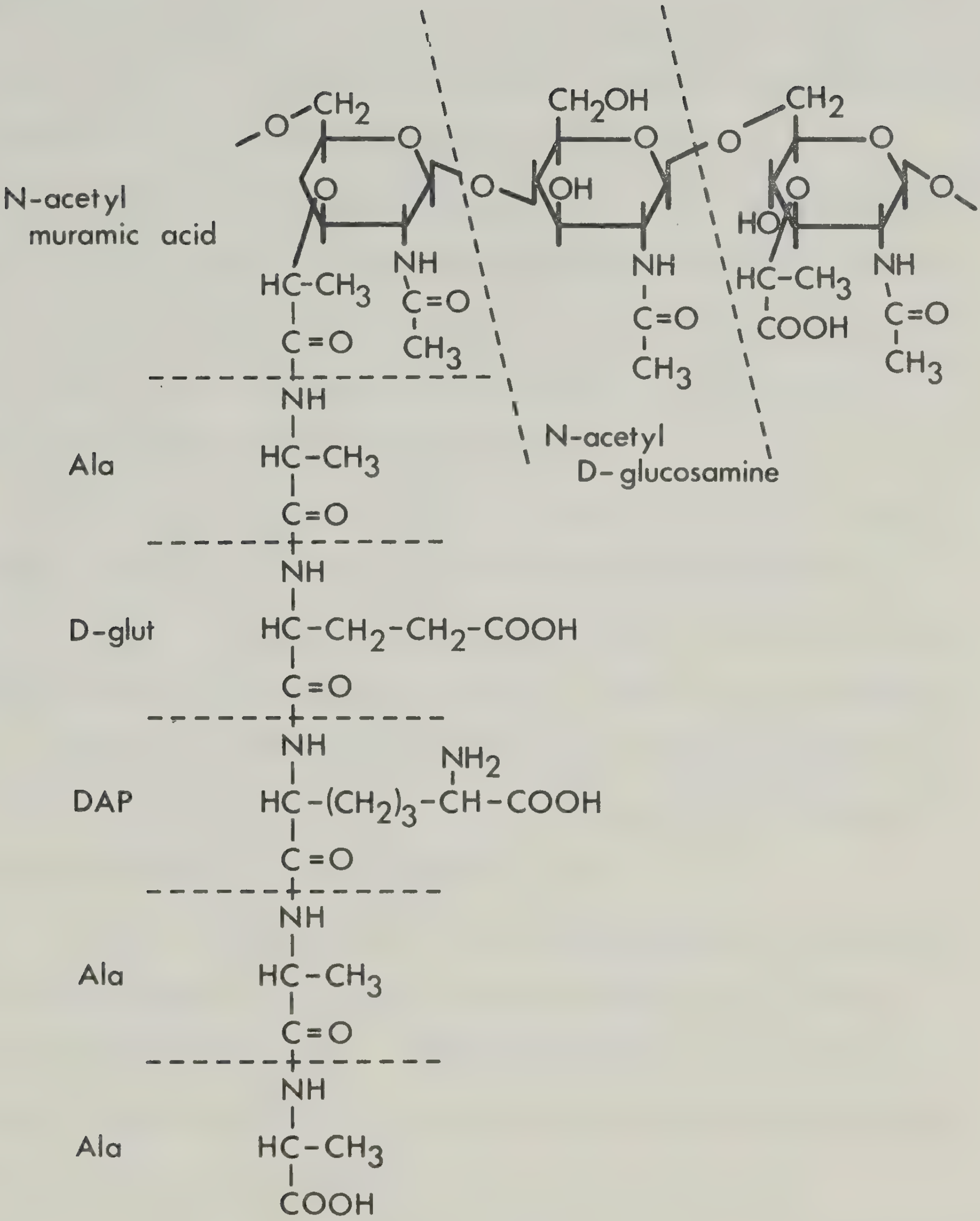

Fig. 3. Hypothetical structure of spore cortex mucopeptide (Murrell and Warth, 1965).

of the DAP which is thought to be involved in side-chain bonding, an increase in the DAP level in the cell would result in an increase in the amount of cross-linking in the cortex. This would tend to agree with the findings of Fitz-James (1963) mentioned above.

c) Calcium-dipicolinic acid complex. This has long been known to be an important factor in the heat-resistance of spores (Powell, 1953; Grelet, 1957; Halvorson, 1957; Church and Halvorson, 1959; Vintner, 1960) and is thought to act by forming a Ca-DPA-protein complex which stabilizes heat-labile proteins.

The original theory of spore resistance was based on the cortex as a permeability barrier surrounding the dry spore coat (Fischer, 1877); Rode et al. (1962) felt that such a barrier was also responsible for the dormancy of the spore. However, work by Black and Gerhart (1962) on the permeability of the spore indicated that water and larger molecules can penetrate the entire spore thus tending to rule out the possibility of an anhydrous core.

The most recent explanation of the resistance of bacterial spores is the "Contractile Cortex" theory of Lewis et al. (1960). They suggested that the cortex contracts around the protoplast thus reducing its water content and since the cortex contains free carboxyl and amino groups, this contraction can be controlled by the concentration of Ca^{++} and/or Ca-DPA. Since proteins are less susceptible to denaturation under conditions of reduced moisture, the protoplast proteins would thus be protected. This theory agrees with most of the findings on spore resistance.

4. Activation

Activation is the process by which a spore is "prepared" for germination. It is a reversible process and an activated spore in conditions unfavorable for germination will revert to the dormant state. Induced activation will result in an increase in the number of spores germinating and a consequent increase in the viable count.

Spores have been activated by heat (Curran and Evans, 1945), water vapor pressure (Hyatt *et al*. 1966), irradiation (Krabbenhoft *et al*. 1966) and by chemical agents (Keynan *et al*. 1965). Of these, heat activation is by far the most effective. The severity of the heat treatment applied will determine whether activation or destruction of the spores will result (see Fig. 4).

The mechanism of activation is thought to be a reduction of the number of disulphide bonds in the cystine rich spore coat protein with a corresponding increase in permeability, thus allowing access of small organic molecules to which the spore was previously impervious. As will be discussed later certain intermediary metabolites which are relatively small molecules can initiate germination. Activation may thus allow germination "triggers" to reach their active sites in the cell.

5. Germination

The overall picture of germination is a breakdown of polymerized murein from the spore cortex (Powell and Strange, 1953) and excretion of approximately equimolar amounts of Ca^{++} and DPA. This is accompanied by a loss of the resistance characteristics of the

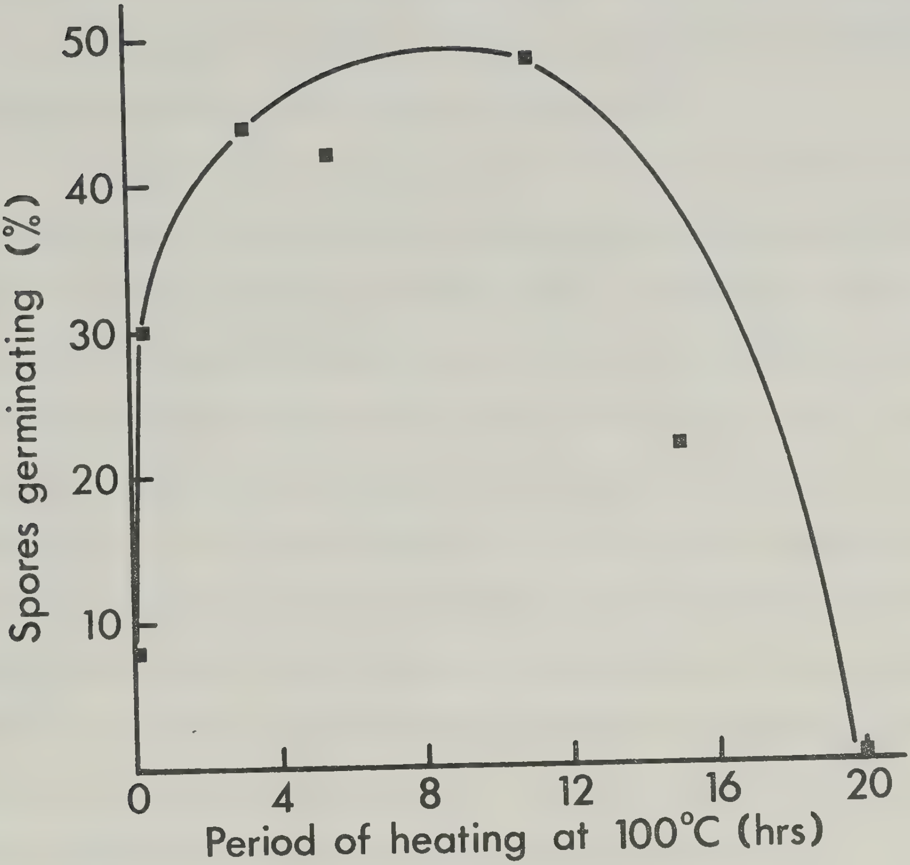

Fig. 4. Effect of heat at 100° on subsequent germination of spores of <u>Bacillus stearothermophilus</u> (Cook and Brown, 1965).

dormant state, a change from phase bright to phase dark and an increase in size of the cell. Unlike activation, germination is irreversible and once initiated, must continue to completion.

Many factors have been shown to induce germination including chelating agents such as ethylenediaminetetraacetic acid (EDTA) and Ca-DPA complexes (Riemann and Ordal, 1961), surfactants (Rode and Foster, 1960), enzymes (Gould and Hitchins, 1963), abrasion and intermediary metabolites such as amino acids (Hills, 1949). The enzymes, abrasion and surfactants are thought to affect the permeability of the spore directly while the chelating agents seem to work by binding preferentially with the spore Ca-DPA. Intermediary metabolites such as amino acids and nucleosides may be considered as metabolic triggers of germination and these are probably the most important in nature. Germination is entirely a degradative process and no new material is synthesized during this phase.

6. Outgrowth of the Germinated Spore

After germination, the outgrowth of the germinated spore into the vegetative cell follows three steps: pre-emergence swelling, emergence, and growth of the new cell.

The germinating spore swells and the coats become more permeable to water and nutrients (Black and Gerhardt, 1962). The amount of swelling varies with the type of spore, small spores such as *B. subtilis* increasing by about 100%, while large spores of the *B. megaterium* type can show increases of 300% or more (Lamanna, 1940; Hitchins *et al*. 1963). During this swelling the cortex becomes large

and spongy.

Development subsequent to swelling consists of the biosynthesis of the vegetative cell. The cell wall of the vegetative cell is thought to originate in the spore cortical membrane, and the spore cytoplasmic membrane is thought to become the cytoplasmic membrane of the cell. These findings have resulted from the examination of ultrathin sections of outgrowing spores by electron microscopy (Hamilton and Stubbs, 1967; Moberly _et al_. 1966; Warth _et al_. 1963b; Takagi _et al_. 1960). During emergence the spore coats of the larger cells seem to be mostly absorbed into the vegetative cell while those of the firmer, small coated cells rupture and are discarded into the medium (Lamanna, 1940; Pulvertaft and Haynes, 1951). The orientation of the emerging cell to the axis of the spore seems to have little relationship to the species of bacterium (Gould, 1962).

Molecular biosynthesis during outgrowth is similar to the lag phase of vegetative cells and consists mainly of repairing the protein synthesizing systems of the spore and an ordered synthesis of proteins (Kobayashi et al. 1965). The initial step is the synthesis of RNA followed by protein synthesis and then DNA synthesis. Growth continues in the form of vegetative cells.

C. The Heat-Inactivation of Spores

The inactivation of spores is usually studied in the laboratory by determining the number of survivors capable of forming colonies after treatment with a lethal agent. This inactivation usually follows a logarithmic order and if the logarithm of the number

of survivors is plotted against the extent of treatment, a straight line will normally result except perhaps at the extremes of the curve. For the purpose of this thesis the only lethal agent considered will be heat.

Stumbo (1965) and Roberts and Hitchins (1969) have given comprehensive reviews of the methods and terminology involved in measuring the resistance of spores. In this dissertation only the "D" value will be mentioned. The "D" value or decimal reduction time is the time necessary at any particular temperature to destroy 90% of the cells. A subscript is used to refer to the temperature of heating, e.g., D_{100° is equal to the time required at 100° to kill 90% of a given bacterial population.

Bacterial spores show a wide range of heat resistance varying from, e.g., _Cl_. _botulinum_ type E ($D_{80^\circ} = 0.6$ min) (Roberts and Ingram, 1965) to the more resistant thermophiles such as _B_. _stearothermophilus_ which has a D_{115° up to 22.6 min (Briggs, 1966). Variation in resistance also occurs between spores of different strains (Roberts and Ingram, 1965) even when they are grown on the same sporulation medium.

Many factors have been shown to affect the heat-resistance of spores but these can be grouped under three headings; (1) the conditions under which the spore is formed, (2) the conditions during heat-treatment and (3) the recovery conditions of the heated spore. These are considered separately.

1. The Conditions Under which the Spore is Produced

a) *Temperature*. It is generally agreed that spores produced at higher temperatures are more heat-resistant than those produced at lower temperatures. Williams (1929) showed this to occur with *B. subtilis* spores and more recent studies have yielded similar findings with another strain of *B. subtilis* (Lechowich and Ordal, 1962), *B. coagulans* (Lechowich and Ordal, 1962) and *B. stearothermophilus* (Gilbert, 1966). The increase in cation/DPA ratio with increased sporulation temperature is thought to play a role in this phenomenon. The explanation may not be quite so simple however since spores of *B. cereus* produced at temperatures between 15° and 41° showed optimum heat-resistance at 30° (Murrell and Warth, 1965). Sugiyama (1951) has also shown spores of *Cl. botulinum* grown at 37° to have greater heat-resistance than those grown at 24°, 29° or 41°.

b) *Composition of the sporulation medium*. The following factors have all been found to have an effect on the heat-resistance of spores.

(i) Inorganic ions. The resistance of *B. megaterium* spores has been shown to depend on the amount of calcium in the medium while *Cl. botulinum* spores have been found to depend on both calcium and iron (Sugiyama, 1951). Amaha and Ordal (1957) found that by varying the concentrations of manganese and calcium in a medium for the production of spores of *B. coagulans*, maximum resistance was obtained using a medium containing 50 ppm $MnSO_4$ and 45 ppm $CaCl_2$.

There is a divergence of opinion as to the effect of phosphate concentration on the resistance of spores. Williams (1929) and Sommer (1930) found the presence of phosphate to increase the

resistance of spores of B. subtilis and Cl. sporogenes while other workers have found the opposite to be the case with B. coagulans (El-bisi and Ordal, 1956) and with B. megaterium spores (Levinson and Hyatt, 1964).

(ii) Organic compounds. Phenylalanine in the sporulation medium was shown by Church and Halvorson (1959) to increase the DPA content and hence the heat-resistance, however L-glutamic and L-proline reduced the heat-resistance of spores of B. megaterium (Levinson and Hyatt, 1964). Yeast extract (Church and Halvorson, 1959) and protein hydrolysates (Tsuji and Perkins, 1962) have been shown to enhance the resistance of B. cereus and Cl. botulinum spores respectively.

The presence of long chain fatty acids increased the heat-resistance of spores of Cl. botulinum (Sugiyama, 1951); the longer the chain of the fatty acid, the greater was the resistance of the spore. Linoleic acid, the only unsaturated acid tested had an adverse effect.

2. The Conditions During Heat Treatment

a) Inorganic ions. Sugiyama (1951) showed that the presence of calcium and magnesium ions in the heating medium lowered the heat-resistance of Cl. botulinum spores. Chelating agents such as EDTA also lowered heat-resistance (Amaha and Ordal, 1957).

b) Water activity. Murrell and Scott (1957) found that spores of Cl. botulinum at an $a_w = 0.8$ showed a thirty thousand fold increase in heat-resistance compared to spores at an $a_w = 1$. B. megaterium and B. stearothermophilus spores under similar conditions showed a 3,000 and 10 fold increase respectively. This is presumably because proteins are

more readily denatured in an aqueous environment than in a dry one.

c) *Lipids*. Bacterial spores suspended in lipid or lipid-like materials are more heat-resistant than those in an aqueous environment (Yesair *et al*. 1946), soybean oil giving greater protection to *B. subtilis* and *B. megaterium* than olive oil, triolein or liquid paraffin. The fats are thought to give protection by isolating the spores in an environment of low a_w.

d) *Carbohydrates*. Weiss (1921) found that *Cl. botulinum* spores in food products of viscous syrup content survived longer than those in light syrup products. Anderson *et al*. (1949) showed that *B. thermoacidurans*, suspended in tomato juice containing different concentrations of glucose or sucrose had heat-resistances proportional to the concentrations of the sugars. Dehydration of the spore by osmosis did not explain this entirely since equimolar solutions of the different sugars gave different increases (Sugiyama, 1951).

e) *pH*. The many reports on the effect of pH of the heating menstruum on the heat-resistance of spores have failed to reach agreement. The most reasonable explanation for this is probably that of Levinson and Hyatt (1960) who pointed out that not only the pH, but the effect of its alteration on the buffer and any other constituents of the menstruum must be taken into account in any study of the effect of pH on heat-resistance.

f) *Antibacterial agents*. Since Anderson and Michener (1950) discovered that the antibiotic subtilin reduced the heat-treatment required to kill spores of *Cl. botulinum* much effort has been put into the search to find substances with a similar effect. In spite of this

effort no compounds as effective as subtilin have been found although nisin and a few subtilin derivatives have been shown to have some effect (Michener *et al*. 1959).

Michener (1955) showed that subtilin does not lower the resistance of spores but is adsorbed onto the heated spore and prevents outgrowth.

3. The Recovery Conditions of the Heated Spore

The effectiveness of any heat-treatment on spores is usually measured by some estimation of the viability of the survivors, most often carried out by measuring their ability to form colonies under defined conditions; this can have a considerable effect on the results. Thus although the recovery conditions cannot affect the heat-resistance of the spores, they must be taken into consideration in any measurement of this resistance.

a) *The composition of the recovery medium*. Heat-treated spores are generally more fastidious in their growth requirements and thus enriched media are often used in their recovery. They are also more sensitive to inhibitors commonly present in media and the addition to the media of any compound which will absorb these such as starch (Olsen and Scott, 1950; Wynne and Foster, 1948), or serum albumin or charcoal (Olsen and Scott, 1950) will improve the recovery of damaged cells.

Sensitivity to sodium chloride, potassium nitrate and sodium nitrite has also been increased by heat-treatment (Roberts and Ingram, 1966).

Examples of media used by various workers during spore

recovery studies have been detailed by Russell (1971).

b) *pH of the recovery medium*. Yokoya and York (1965) found that the composition of the medium affected recovery of *B. stearothermophilus* spores at pH 7 but not at pH 6.5. Cook and Brown (1965) found that both heated and unheated spores of *B. stearothermophilus* were affected by the pH of the recovery medium, the highest counts for untreated spores being obtained on a medium of low pH (5.3) and for treated spores at pH 7.3.

c) *Germinants*. During heat-treatments the requirements for germination may be altered (Campbell *et al*. 1965), and thus the presence of germinants in the recovery medium can enhance the recovery of damaged spores. Busta and Ordal (1964) for example found this to be so with Ca-DPA; also L-alanine has been shown to be the most effective for a wide number of species by many workers. Heat activation of spores of *B. megaterium* at 60° for 10 min allowed germination over a wider range of compounds than without this treatment (Levinson and Hyatt, 1962). This was thought to occur because germination is a biophysical process as well as a metabolic one with heating possibly rearranging the molecular structures of the spore.

The germination requirements of *Clostridium spp*. are much less well understood and appear to be more complex. Roberts and Hobbs (1968) found difficulty in stimulating the germination of several *Clostridium* species, germination occurring most frequently in complex media.

d) *The temperature of incubation during recovery*. Little information is available on the effect of incubation temperature on the

recovery of heat-treated bacterial spores, but the results obtained so far suggest that some species recover better at temperatures below the optimum for that of unheated spores. Sugiyama (1951) showed that germination of *Cl. botulinum* spores was greatest at 29° and 24° and Futter and Richardson (1970) found heat-damaged spores of *Cl. welchii* gave optimum recovery at 27° as did Williams and Reed (1942). Edwards *et al*. (1965) showed that *B. subtilis* spores subjected to 'Ultra High Temperature' treatment (150°/2 sec) showed greater recovery at 32° than at 45°; this was opposite to the results they obtained with untreated or slightly heated spores. Cook and Gilbert (1968) obtained maximum colony counts of *B. stearothermophilus* spores heated at 115° for various times on incubation at 45 - 50° whereas unheated spores had a maximum recovery at 50 - 65°. They suggested that this might be caused by the production of a heat-sensitive mutant.

Any estimation of the effectiveness of heat-treatment in the destruction of bacterial spores usually depends on a measurement of the viability of the survivors. This is usually done by a cultural procedure involving a colony count. As has been pointed out the recovery of damaged cells is more susceptible to factors in the environment than is that of undamaged cells, therefore, in thermal death studies, recovery conditions must be carefully chosen. Of all the factors affecting the recovery of damaged spores, temperature is perhaps the most easily controlled and yet has received little consideration.

This work was therefore undertaken to study the effect of incubation temperature on the recovery of bacterial spores subjected to a variety of heat-treatments.

II. EXPERIMENTAL

A. The Influence of Incubation Temperature on the Recovery of Bacillus subtilis Spores

Little systematic work on the effect of incubation temperatures on spore recovery has been carried out perhaps mainly because of the difficulty of incubating large numbers of plates over a wide range of temperatures. Cook and Gilbert (1968) used 5 different temperatures differing by up to 12 degrees, Futter and Richardson (1970) incubated at temperatures of 4 degree increments, Sugiyama (1951) used 4 different temperatures, while Edwards *et al*. (1965) studied recovery at only two incubation temperatures.

Thus in the present study more extensive information was sought by carrying out colony counts over a more comprehensive range of temperatures. This was achieved by making colony counts in roll tubes (Astell and Co., Brownhill Rd., London, S.E. 6) incubated in a specially designed temperature-gradient incubator.

1. Materials and Methods

a) *The design of the temperature gradient incubator*. Design criteria of the incubator included uniformity of temperature of wall of the sample well (so that the solid medium in the roll tube had a uniform temperature); uniformity of temperature of sample wells in any given row for replicate experiments; and linearity of the temperature gradient so that intermediate temperature measurements would not be

required. Readily available heated and refrigerated baths were used as constant temperature heat source and heat sink.

The heat conducting section of the incubator (Fig. 5) consisted of a heavy aluminum slab with heating and cooling sections at opposite ends and 21 blocks 1 1/2 in thick each having 6 sample wells situated at intervals of 1 3/4 in along the bar. The spaces between the sample blocks were filled with expanded polystyrene. The incubator was surrounded by a 1 in layer of insulation, a 1/4 in thick aluminum plate, another layer of insulating foam, and the whole enclosed in a 3/4 in plywood box (Plate I). The aluminum plate front, bottom, back and lid was equipped with flow channels for the heating and cooling fluids at the hot and cold ends, so that a temperature gradient was set up in the plate itself. Each row of sample wells had a cover of polystyrene specially shaped to fit over the tops of the roll tubes. Use of separate covers for each row enabled sample tubes to be added to or withdrawn from the incubator without exposing the whole set of sample wells (and tubes) to ambient temperature.

The sample blocks were separated so that there was no heat flow between the holes to give rise to a temperature drop from one side of the hole to the other in the direction of heat flow. This method of obtaining a uniform temperature of the sample well wall was considered to have advantages over that of Oppenheimer and Drost-Hansen (1960) who used agitation of the temperature gradient bar to rotate the tubes in the sample well, and also of Selwyn (1961) who constructed an incubator of a series of brass blocks separated by 1.5 mm plates of Tufnol (an insulating material). The disadvantage of the Oppenheimer

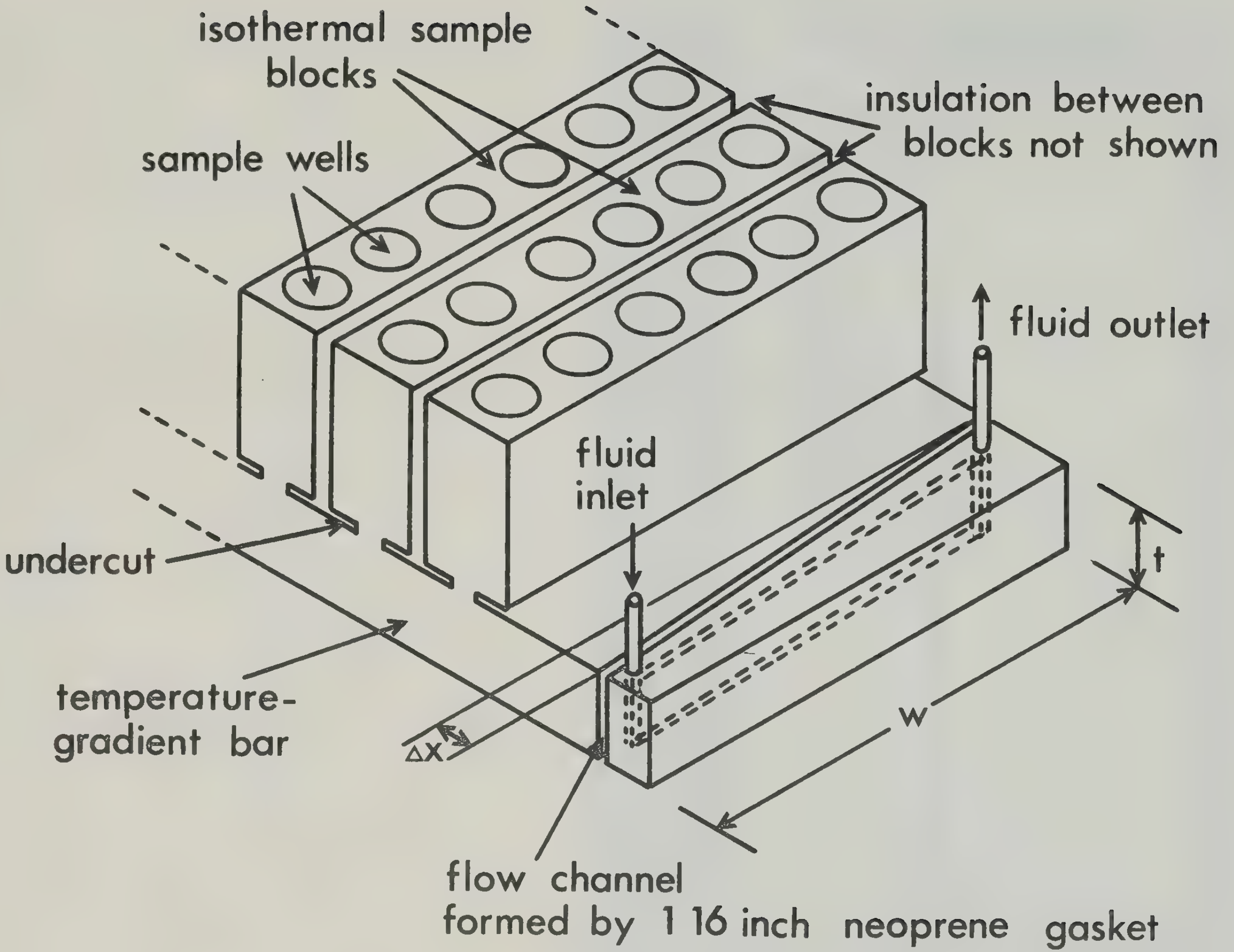

Fig. 5. Details of construction of the bar and sample blocks of the temperature gradient incubator.

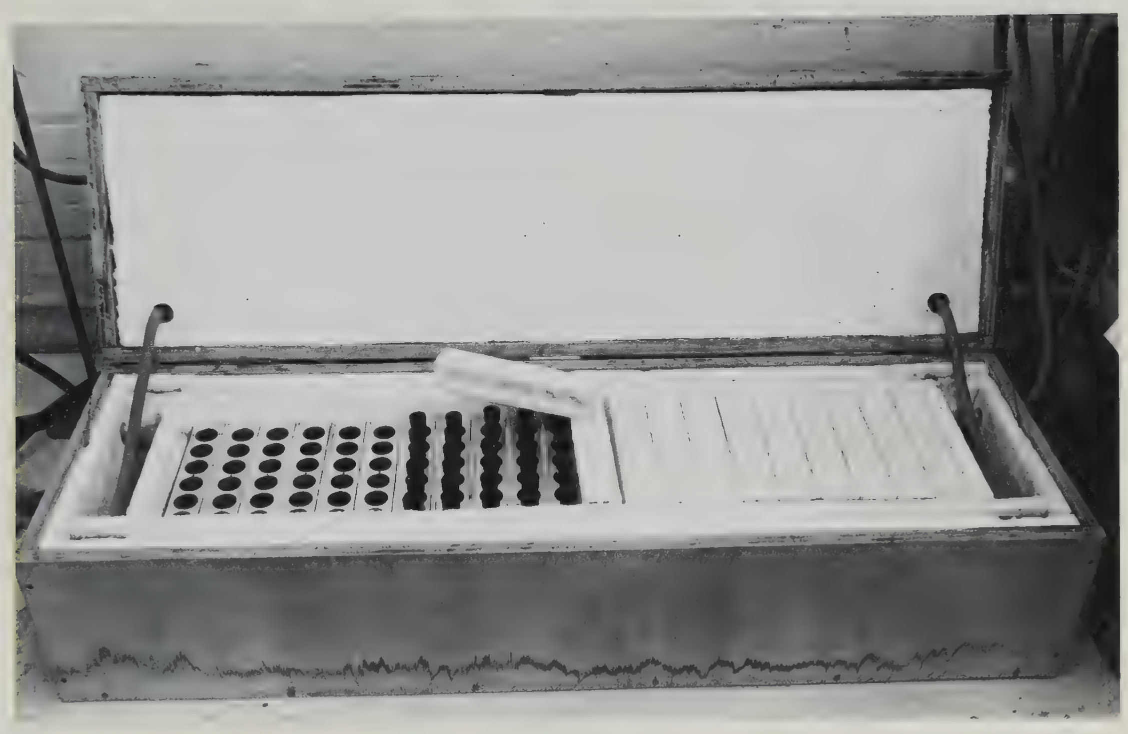

Plate I. The Temperature Gradient Incubator

and Drost-Hansen method is that it increases the complexity and cost of the equipment. Selwyn's method gives a much lower heat flow than occurs in a continuous bar. This results in a slower rate of approach to steady temperature in the incubator and also makes it more susceptible to non-linearity of the temperature gradient by loss of heat through the insulation surrounding the bar.

The sample blocks were undercut to improve the uniformity of temperature of the wall near the bottom of the sample wells. Temperatures computed on an IBM computer using a Fortran G program indicated that this modification reduced the difference between the hottest and coldest points at the bottom of the sample well from about 20% of the temperature difference between consecutive rows of holes to less than 2%.

Heat transfer to and from the hot and cold end of the gradient bar was effected by pumping the liquids, from the constant temperature baths, through channels in the ends of the bar. The temperature of the heating fluid dropped as it passed across the bar and the channel was therefore cut at an angle so that the drop in temperature of the fluid as it flowed across the bar was equal to the drop in temperature resulting from the temperature gradient along the bar. The mass flow rate F of the heating fluid (specific heat S) is given by the equation

$$F = kwt/S\Delta x,$$

where k is the thermal conductivity of the gradient bar, w and t are the width and thickness respectively of the gradient bar, and Δx is the distance between the fluid inlet and outlet measured in the direction

of the temperature gradient as shown in Fig. 5.

Heat losses through the insulation surrounding a temperature gradient incubator when used at temperatures much above or below ambient temperature give a non-linear temperature gradient. Calculations and measurements showed that the deviations from linearity with an incubator insulated with 1 in of expanded polystyrene and 3/4 in of plywood would be of the order of 1° when used with the hot end at about 55° and the cold end at about 20°. Calculations were then done to determine the deviations from linearity with a temperature gradient aluminum jacket. The maximum deviation from linearity when used between 55° and 20° (with a surrounding temperature of 20°) was calculated to be less than 0.08° and measurements on an experimental run gave a maximum deviation of 0.13°. The calculated and experimental deviations for the jacketed incubator are shown in Fig. 6 along with experimental deviations for the unjacketed incubator (i.e., the incubator with 1 in of polystyrene foam and 3/4 in of plywood). The irregularities in the graphs of measured deviation are caused partly by uncertainties in the measurement of temperatures (±0.03°) and partly by small inaccuracies in the placement of spacer strips between the bar and the sample blocks. A better method of providing undercut blocks would be to mill the grooves across the main bar, and position the sample blocks on the elevated portions.

Some difficulties were encountered in pumping water, especially hot water, at a constant rate across the ends of the bar. These were overcome however by delivering the water via a constant head system.

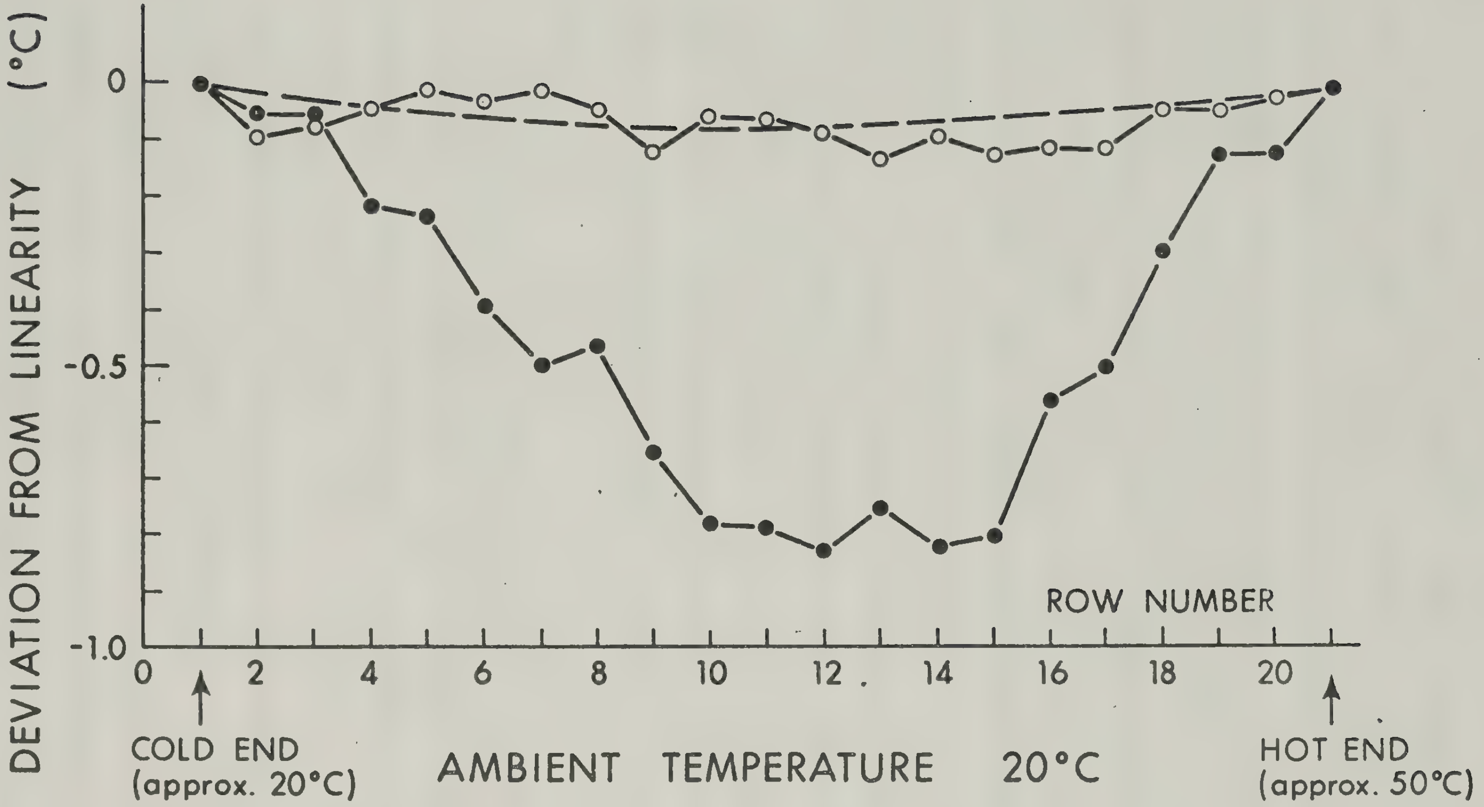

Fig. 6. Deviation from linearity for the temperature gradient incubator. Broken line, calculated deviation for incubator with a temperature gradient jacket; open circles, actual deviation for incubator with temperature gradient jacket; filled circles, actual deviation for incubator with no jacket.

The form of the incubator, which has proved quite satisfactory over a period of about 2 years, could be varied considerably to meet specific requirements of a wide range of studies. It can be used with liquid media, and could be shaken if necessary as is the incubator described by Oppenheimer and Drost-Hansen (1960) and that produced by the Toyo Kagaku Sanyo Co. Ltd. (No. 7, 3-Chome, Hon Cho, Nihonbashi, Chuoko, Tokyo, Japan). The incubator can be used over wide or narrow temperature ranges, with the maximum and minimum temperatures being limited only by the capacities of the heat source and heat sink. The print-out of the computer program and results are given in Appendix A.

b) Bacterial strain. The organism used throughout these studies was Bacillus subtilis strain 8057 NCIB-8057 (ATCC 9524; NRRL B-314), NCIB, 1955. This strain was chosen because it has excellent spore-producing capabilities and has been extensively studied in this laboratory.

c) Preparation of the spore suspensions. The spore suspensions were prepared by the method used by Nath (1968). Nutrient agar (Difco Laboratories, Detroit 1, Michigan, U.S.A.) containing 1 mg/l of $MnSO_4$ and 0.5 g/l of $CaCl_2$ was dispersed in Roux bottles in 100 ml amounts. After inoculation with 1 ml of an actively growing culture of Bacillus subtilis 8057 in trypticase-soy broth (TSB), (BBL, Becton, Dickinson and Co., Cockeysville, Maryland 21030, U.S.A.), the bottles were incubated at 37° for 72 h. The spores were harvested with a sterile glass scraper in sterile distilled water and concentrated by centrifuging at 10,000 rev/min (12,000 g) for 1 min after adding a drop of Tween 80 to encourage sedimentation.

The lysozyme method of Finley and Fields (1962) was used to purify the spore suspension. The spores were suspended in 50 ml distilled water containing 0.5 mg/ml of lysozyme and incubated at 52° for 2 h with stirring. The suspension was then washed by centrifugation at 3000 rev/min (1465 g) for 20 min. After resuspending in sterile distilled water, the spores were shaken with glass beads for 2 h at 4° to break up any clumps. The purification of the suspension was completed by centrifuging for 20 min at 3000 rev/min and for the same time at subsequent speeds increasing by 500 rev/min up to 8000 rev/min. On suspension in 50 ml sterile distilled water, a stock suspension yielding a viable count of c. 6 x 10^9 colonies/ml was obtained. This stock suspension was stored in a screw capped bottle at 4° and was renewed at intervals of about 1 month.

For each experiment 1 ml of the stock spore suspension was diluted in sterile distilled water to give a viable count of c. 2 x 10^8/ml on Plate Count Agar (PCA) (Difco) and this was diluted further with an equal amount of sterile 2 M phosphate buffer pH 7. This gave a working suspension of c. 10^8 colonies/ml in 1 M phosphate buffer at pH 7.

d) Heat treatment. Two ml amounts of the working suspension were introduced into 2 ml sterile thin glass ampoules (Wheaton Glass Company, Millville, New Jersey, U.S.A.) by means of a graduated 3 ml disposable syringe (America Hospital Supply Division of AHSC, Evanston, Illinois 60201, U.S.A.) and after sealing, the ampoules were completely immersed in an oil bath at the appropriate temperature for selected times. On removal they were cooled immediately by placing in ice

water.

e) Colony counts. Serial dilutions of the heat-treated spores were made in 9 ml amounts of quarter strength Ringer's solution (Oxoid Limited, London, S.E. 1) and 0.2 ml of suitable dilutions added to 4.5 ml of the molten media at 45° in roll tubes. The tubes were cooled under running water while being rotated on an Astell roller (Astell and Co., Brownhill Road, London, S.E. 6) so that the medium formed a thin layer around the inside of the tubes which were then placed upright in the incubator. Three replicate tubes of each of two dilutions were incubated at each temperature.

The temperature gradient incubator was arranged with the cold end at c. 15° and the hot end at c. 56°. Actual temperatures for each of the tubes were determined by measuring accurately the temperature of each end of the bar and, assuming the gradient to be linear, reading off the values of the intermediate temperatures from a graph of temperature vs. distance along the bar.

The colonies formed were counted every 24 h for 7 days after which no increase in colony count was observed at even the lowest temperature. The highest colony count for each tube was taken as the actual count.

Counts were made on both PCA and Brain Heart Infusion Agar (BHIA) (Difco) in which the concentration of agar had been increased to 2% (w/v) to compensate for the dilution of the medium by the inoculum.

f) Standardization of colony counts. The results of the colony counts were standardized by carrying out a Standard Plate Count (SPC) on

the working suspension before each run and calculating the factor which would convert this to an SPC of 10^8 colonies/ml. Each colony count obtained on that run was then multiplied by this factor before plotting. This allowed colony counts obtained on different runs to be compared as if the SPC of each of the working suspensions was 10^8/ml.

2. Results

The colony count data for this and subsequent sections are given in Appendix B.

In the first experiment suspensions were subjected to heat treatments at 95° for periods from 0 to 70 min in 5 min increments, and then inoculated into roll tubes and incubated at temperatures from 16° to 56° in 2° intervals. The results expressed in Fig. 7 show only the curves for heat-treatment at 10 min intervals. This allows the graph to be presented more clearly. The curves not plotted were of the same type as those shown. In PCA undamaged (not heat-treated) spores gave similar recoveries over the range of incubation temperature from 16° to 50° but showed a rapid decrease in recovery above this range. With more severe heat-treatments the range of incubation temperature giving the maximum degree of recovery for any given heat-treatment gradually diminished until, after treatment at 95° for 70 min, this was narrowed to 24 - 32° (Fig. 7). Incubation in BHIA gave similar results (Fig. 8).

In industrial practice, temperatures used in heat-treatments are usually higher than 95°, therefore to determine whether the same temperature dependency occurred under practical conditions the recovery experiments were carried out after treatment at a higher temperature.

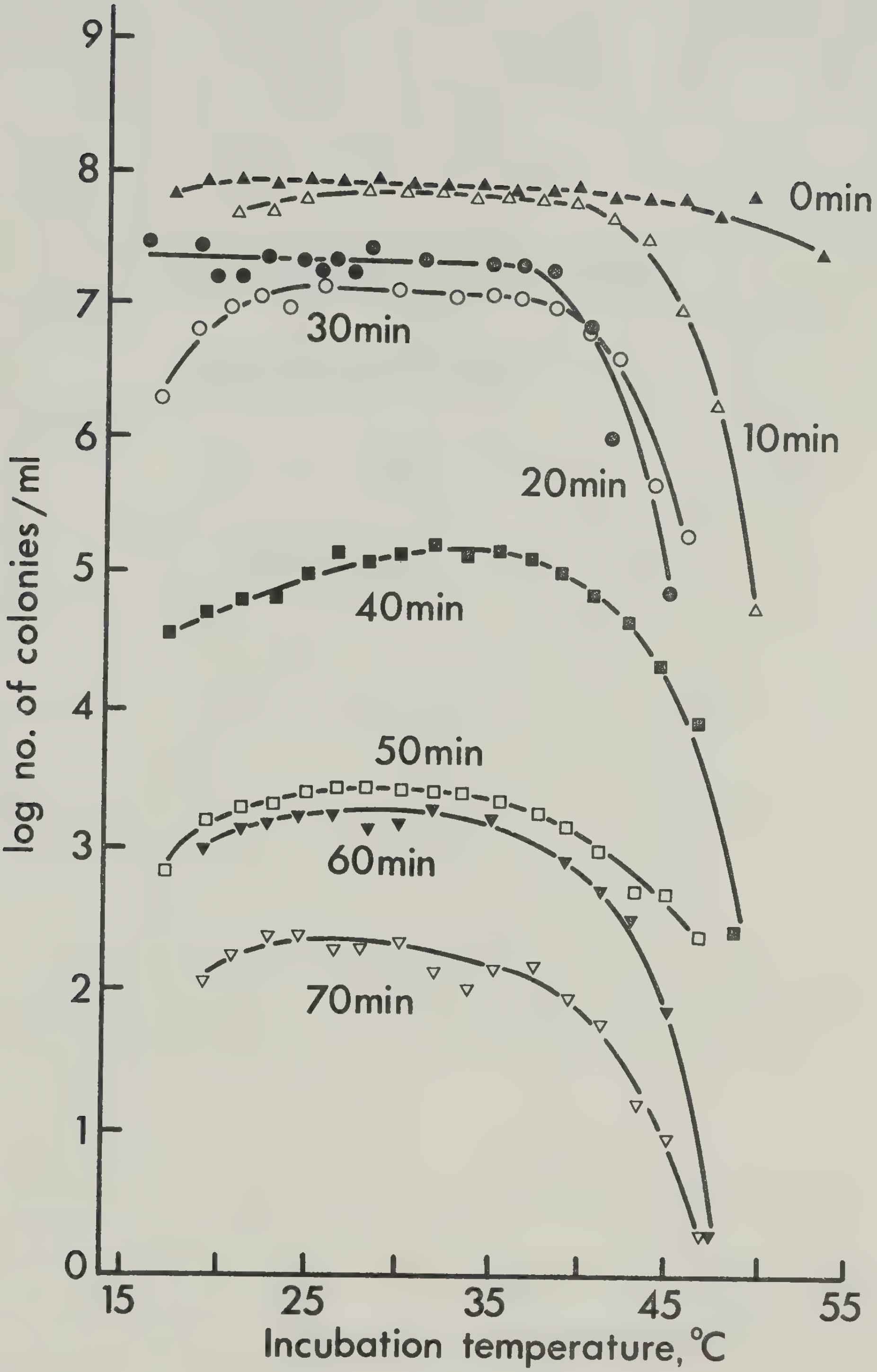

Fig. 7. The effect of incubation temperature on the recovery in Plate Count Agar of <u>B</u>. <u>subtilis</u> 8057 spores after treatment at 95° for different times.

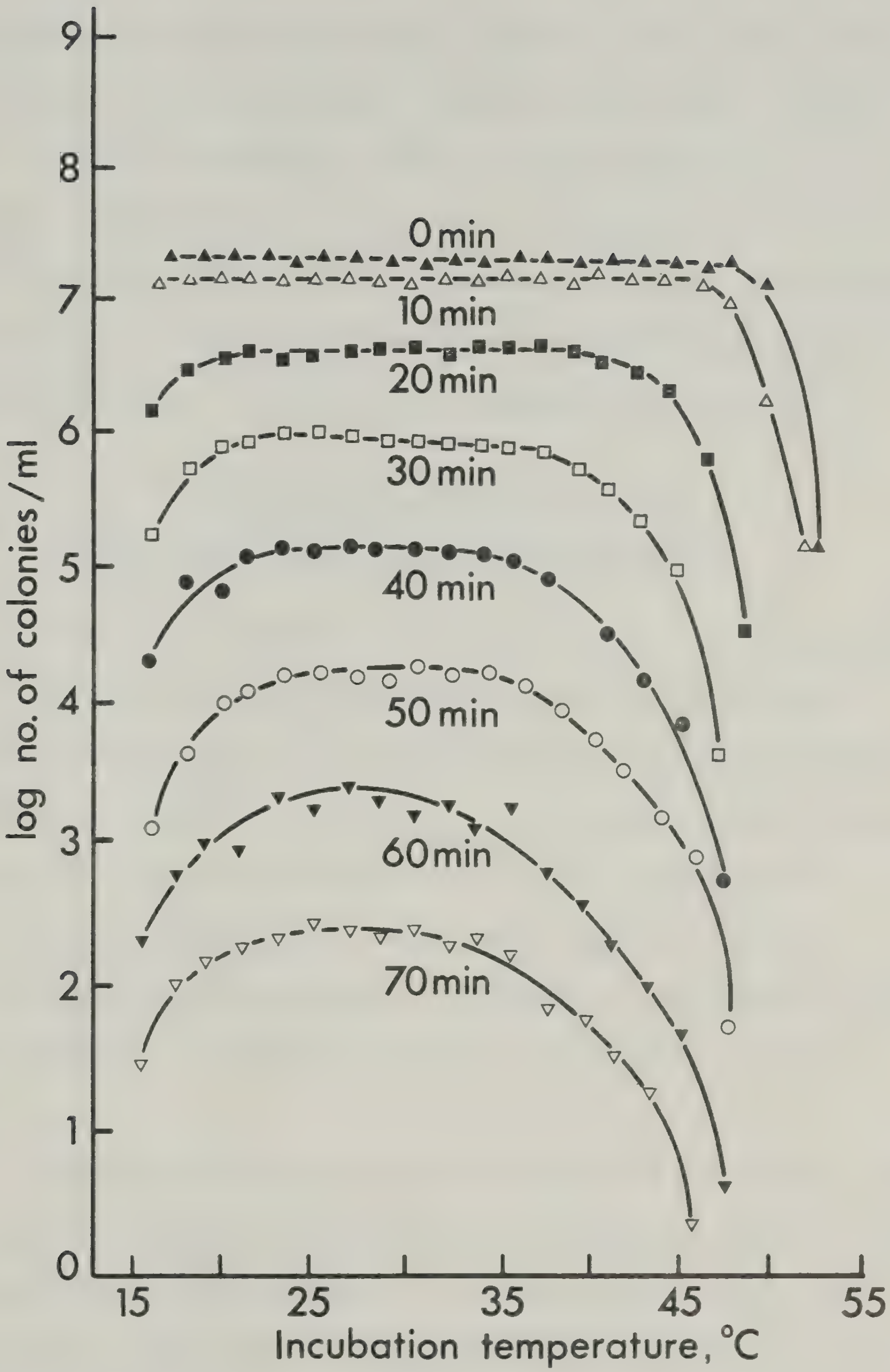

Fig. 8. The effect of incubation temperature on the recovery in Brain Heart Infusion Agar of B. subtilis 8057 spores after treatment at 95° for different times.

At a temperature commonly used in food processing (115°), the viable count of heated spores was reduced so markedly (from 1.3 x 10^8/ml to 0/ml in 4 min) that this aspect could not be investigated with the experimental techniques used in this study. Treatment at 105° (Fig. 9) for different times however showed an incubation temperature dependency pattern similar to that of treatment at 95° suggesting that the same phenomenon might occur with even higher treatment temperatures.

B. The Influence of Temperature on the Germination of Bacillus subtilis 8057 Spores

The recovery of spores as assessed in section A includes the steps in development from the dormant spore to a visible colony viz. germination and outgrowth. Since the incubation temperature has been shown to have a considerable influence on the recovery of heat-treated spores, it was decided to investigate the effect of temperature on the germination of *B. subtilis* spores before and after heat-treatment. This it was hoped would provide some information on the reason for the observed increase in incubation temperature sensitivity with heat-treatment.

Sussman and Halvorson (1966) have discussed the criteria which have been used to measure germination rates of bacterial spores. These are direct microscopic examination, loss of resistance to heat and chemical agents, measurement of changes in form and structure, gain in stainability, loss of spore components and the increase of metabolic activity. Although all of these changes appear at approximately the same time during germination, heat-resistance is thought to be the

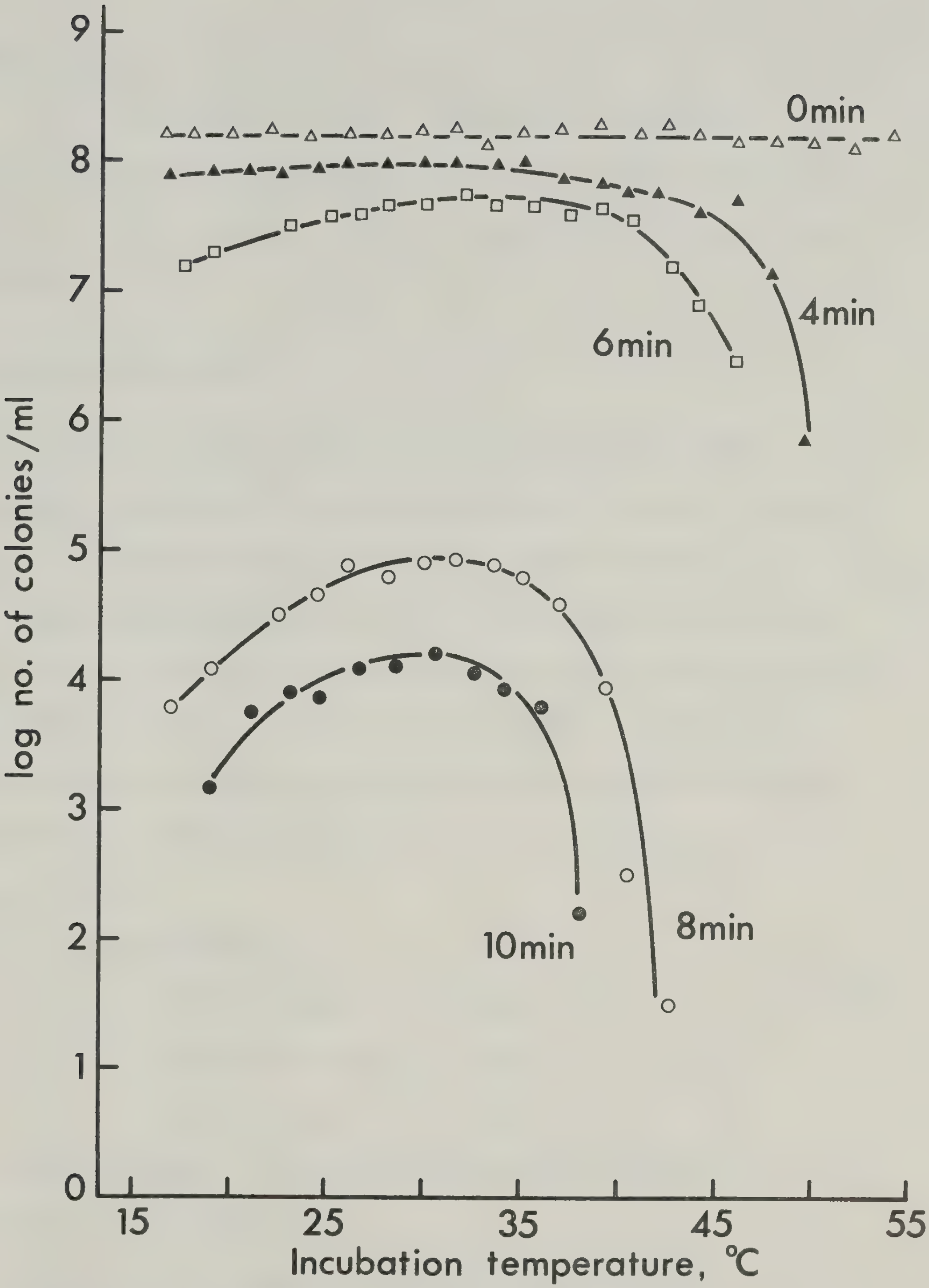

Fig. 9. The effect of incubation temperature on the recovery in Plate Count Agar of B. subtilis 8057 spores after treatment at 105° for different times.

critical change delineating germination.

In this study the rates of germination at different temperatures of heated and unheated spores were followed by both the reduction in optical density at 625 nm (OD_{625}) and the loss of heat-resistance.

1. Materials and Methods

a) Spore suspensions. The suspension of *B. subtilis* 8057 spores was prepared in the manner described in section A to give a final SPC of c. 1 x 10^8 colonies/ml in 1 M phosphate buffer at pH 7.

b) Germination medium. The medium used in germination studies has been shown to have a marked effect on the measurement of germination rates (Thorley and Wolf, 1961). To allow comparisons between the results obtained in section A and the germination rates in this study, the germination medium used contained the same constituents as PCA without the agar.

The composition was:

Bacto-Tryptone	5 g/l
Bacto-Yeast Extract	2.5 g/l
Bacto-Glucose	1 g/l

c) Heat-treatment. The heat-treatment was carried out in the same fashion as in section A.

d) Measurement of changes in OD_{625} during germination. A 3 ml cell containing 3 ml of sterile germination medium was placed in a thermostatically controlled cell compartment of a Beckman DB-G spectrophotometer (Beckman Instruments Inc., Palo Alto, Calif., U.S.A.).

After the required temperature had been reached, 0.05 ml of the spore suspension was added, the cell was covered with a sterile paraffin film, inverted twice to obtain a homogeneous mixture, and the rate of change at OD_{625} with time was recorded on a Beckman 10" recorder using sterile medium as a blank.

e) Measurement of loss of heat-resistance during germination. One ml of the treated suspension was added aseptically to 99 ml of the germination medium in a 250 ml conical flask which was being shaken at the required temperature. At selected time intervals 1 ml portions of the flask contents were withdrawn and added to a screw-capped test tube containing 9 ml quarter strength Ringer's solution at 80° in a water bath. After 10 min at 80°, the tube was cooled immediately in ice water and further serial dilutions made in quarter strength Ringer's solution. Standard Plate Counts were made and the colonies counted after incubation for 24 h at 30°.

2. Results

The rate of germination of unheated spores in the germination medium, shown by a reduction in OD at different temperatures was found to increase from 35° to 40° and then to decrease gradually until at 50° no reduction in OD was observed (Fig. 10). The optimum germination rate was in the region of 40°.

Spores heated at 95° for 20 min germinated more slowly at most temperatures than the untreated spores; however, they still showed some ability to germinate up to 52.5° at which temperature the unheated spores could not germinate (Fig. 11). Spores which were subjected to

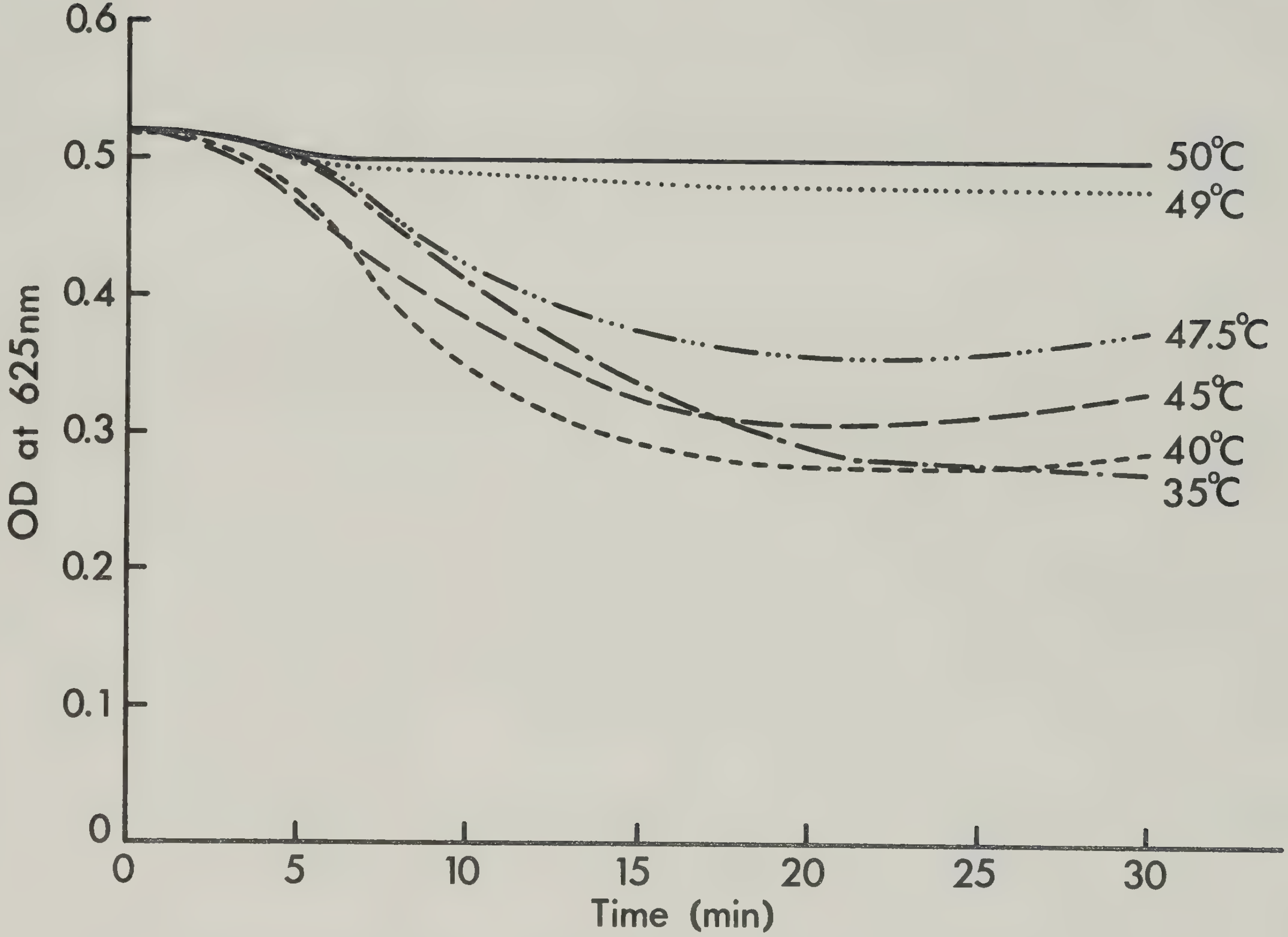

Fig. 10. The effect of different incubation temperatures on the germination of untreated *B. subtilis* 8057 spores.

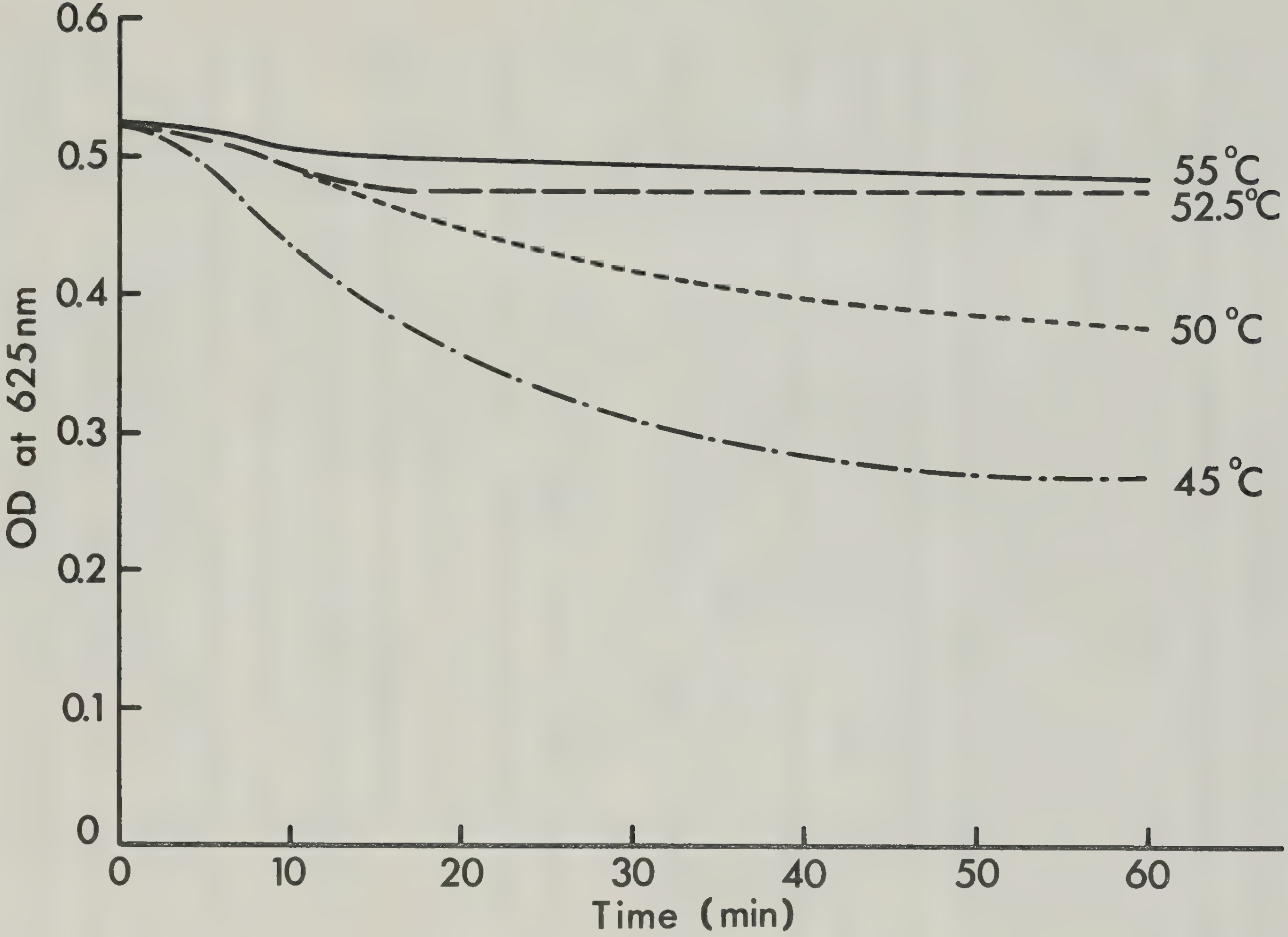

Fig. 11. The effect of different incubation temperatures on the germination of _B_. _subtilis_ 8057 spores after treatment at 95° for 20 min.

treatments more severe than 20 min at 95° showed no measurable decrease in OD on incubation. This could be because the number of survivors after the more severe treatment would be fewer; furthermore there would be a higher percentage of damaged spores and these would germinate more slowly than undamaged cells.

Because the drop in OD became so slow after heat-treatment a further attempt was made to assess the effect of temperature of incubation on the rate of germination of spores using loss of heat-resistance as the criterion of germination. Dormant spores were subjected to heat-treatments at 95° of 0, 30 and 60 min, incubated in the germination medium at different temperatures of 20° and 30°. The reduction in the number of heat-resistant spores was measured through a 24 h period.

Very little can be deduced from the results obtained on incubation at 20° (Fig. 12). Germination rates with all three treatments were very slow, the suspension heated at 95° for 30 min germinated slightly faster than the other suspensions.

The results obtained by incubating at 30° the suspensions subjected to the three heat-treatments are given in Fig. 13. The untreated suspension gave a very fast initial germination rate, the 30 min treated suspension was slightly slower and the 60 min treatment slower still. With the unheated spores and spores treated for 30 min an increase in the numbers of dormant spores appeared after 2 1/2 h and 6 h respectively. This was most likely due to the formation of new spores and since there is no simple method of distinguishing between the original suspension and spores formed during the experimental run

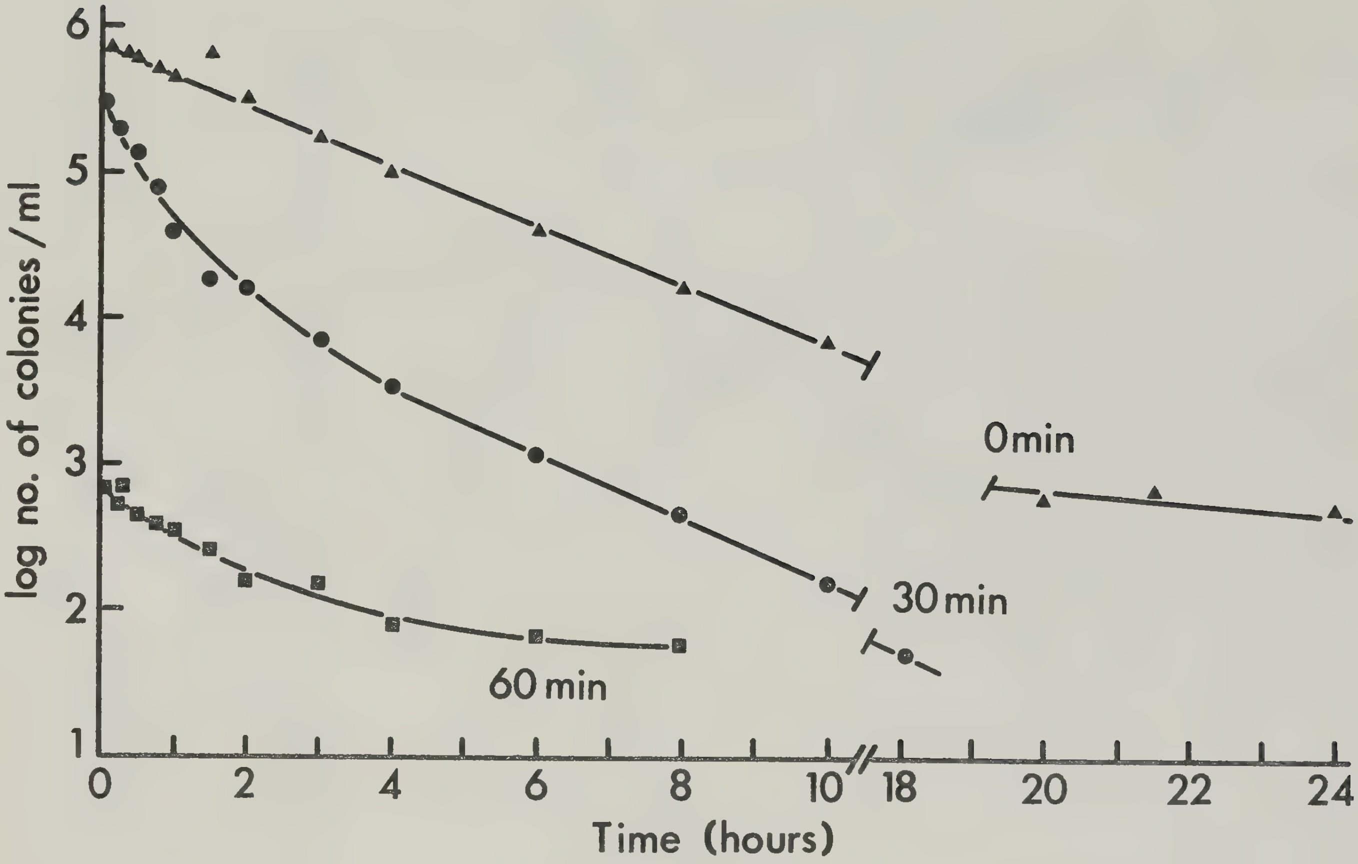

Fig. 12. The germination of _B. subtilis_ 8057 spores at 20° after treatment at 95° for different times.

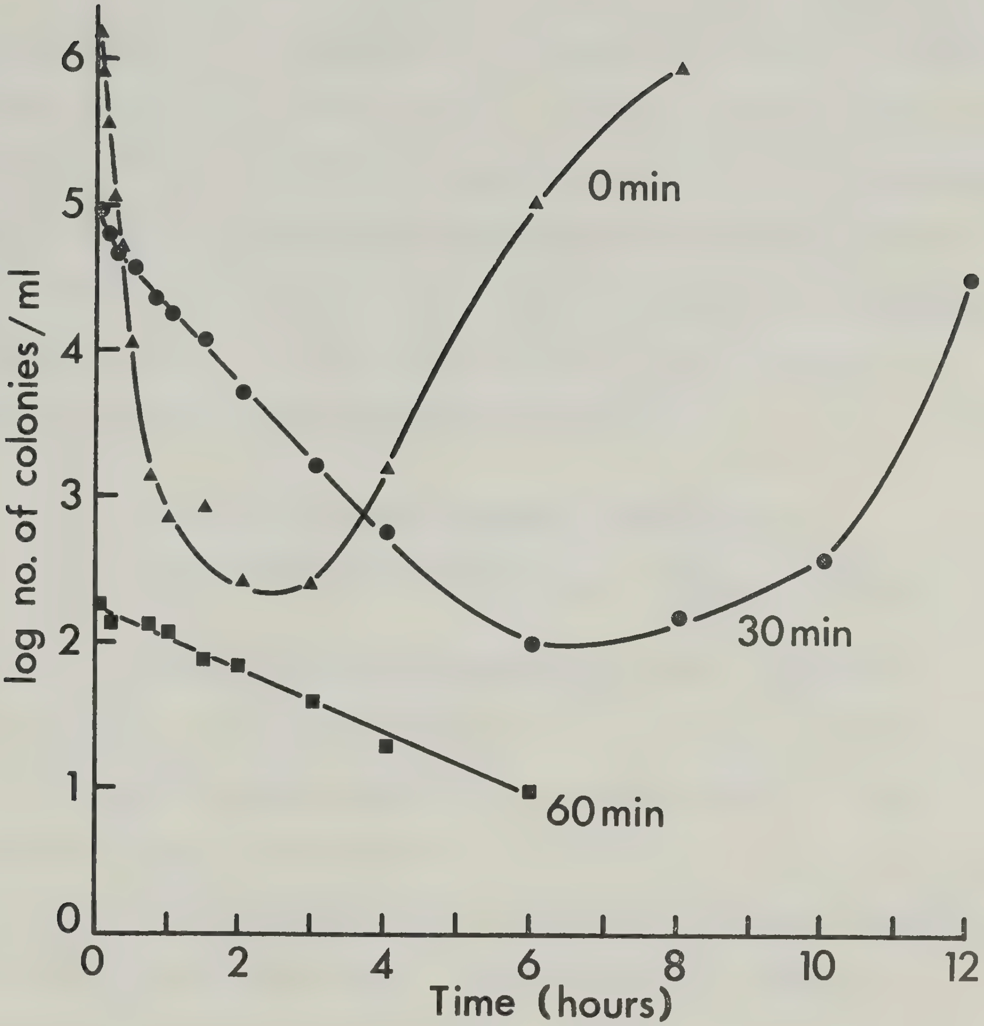

Fig. 13. The germination of B. subtilis 8057 spores at 30° after treatment at 95° for different times.

the data obtained must be interpreted with care.

It was originally intended to carry out these studies by incubation at 20°, 30°, 40° and 50° to observe the effect of temperature on spores heated for different times. The initial rate of germination at 30° however was so rapid that comparisons of rates of this magnitude would have been very difficult; furthermore the formation of fresh spores before all the spores in the original suspension had germinated, confused the study. It was decided therefore that little was to be gained by continuing with this aspect of the work.

C. The Use of Density Gradient Centrifugation for the Separation of Germinated from Ungerminated Spores

To study the effect of temperature on the outgrowth of germinated spores into vegetative cells it is necessary to find a method of separating germinated from ungerminated spores. During germination, changes occur in the size and density of spores suggesting that density gradient centrifugation (DGC) might be a useful technique for effecting such a separation. Tamir and Gilvarg (1966) used renografin, a high density liquid which they had shown to have no effect on spores, to separate the spores of *B. megaterium* from vegetative cells and also to separate spore suspensions into lighter spores and heavier spores.

This section describes studies on the fractions of a suspension of spores of *B. subtilis* obtained by DGC on renografin and sucrose.

1. Materials and Methods

a) Preparation of the spore suspension. The spore suspension was prepared as described in section A.

b) Preparation of gradients. The gradients were prepared with a Buchler gradient maker (Buchler Instruments Inc., Fort Lee, N.J., U.S.A.) in 1 x 3 1/2 in cellulose nitrate tubes, for the Spinco SW27 rotor (Beckman Instruments Inc., Palo Alto, Calif., U.S.A.).

(i) Sucrose. Gradients with density limits of 1.02 - 1.23 g/ml were prepared by using 19.5 ml of a 5% w/v solution and 18.5 ml of a 50% w/v solution.

(ii) Renografin. These gradients were formed by using 19 ml of distilled water and 16 ml of renografin to give density limits of 1.0 - 1.4 g/ml. The renografin was supplied by E.R. Squibb & Sons Ltd., Cote de Liesse Rd., Montreal 379, P.Q., Canada in 70% aqueous solution referred to here as pure renografin.

c) Centrifugation and sampling. A volume of 0.2 ml of the spore suspension was layered on the surface of the gradients in a 1 x 3 1/2 in cellulose nitrate tube in such a manner as to prevent mixing. Each tube was then centrifuged on a Beckman L2-65B ultracentrifuge for 30 min with the renografin and for 10 min with the sucrose gradients both at 15,000 rev/min (29,300 g).

All gradient tubes were fractionated by upward displacement of the tube contents using 50% sucrose or pure renografin. The displacement solutions were dyed blue to allow the displacement to be followed visually. The contents of the tube were passed through a continuous flow cell in a Beckman DB-G spectrophotometer and the

absorbance at 625 nm recorded before fractions were collected at 1/2 min intervals.

d) Measurement of O.D. changes. The spores were suspended in TSB to a suitable O.D. at 625 nm and the decrease in O.D. at 37° followed in a thermostatically controlled cell compartment of the Beckman DB-G spectrophotometer, using sterile TSB as a blank.

e) Microscopic examinations. Spores were stained with malachite green, and observed before and after staining in the phase contrast and optical microscopes.

2. Results

Centrifugation of the stock suspension of spores of *B. subtilis* 8057 in both sucrose (Fig. 14) and renografin (Fig. 15) gradients gave two distinct bands which were readily separated upon fractionation of the gradient tube contents. Microscopic examination of Gram-stained preparations from both bands of each gradient confirmed the absence of vegetative cells. Phase contrast microscopy indicated that the spores in the less dense band were predominantly phase dark while the heavier band consisted of predominantly phase bright spores. Although similar band patterns were obtained in both gradient media, the resolution obtained in renografin was far superior to that on sucrose. This may be due in part to the differences in density of the two gradients.

Figs. 16 and 17 depict the results of studies to determine germination of spores from the different bands after separation from the gradient. In each case the heavier band exhibited rapid O.D.

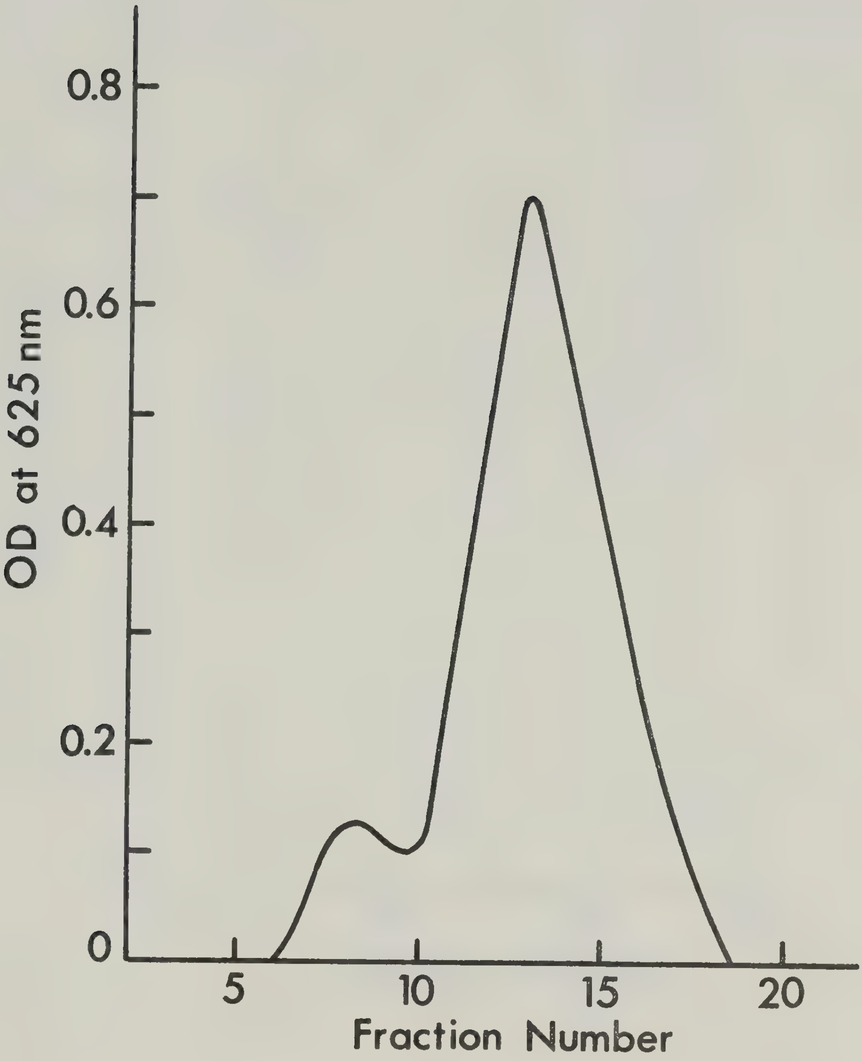

Fig. 14. Fractionation of spores of _B_. _subtilis_ 8057 by density gradient centrifugation in sucrose.

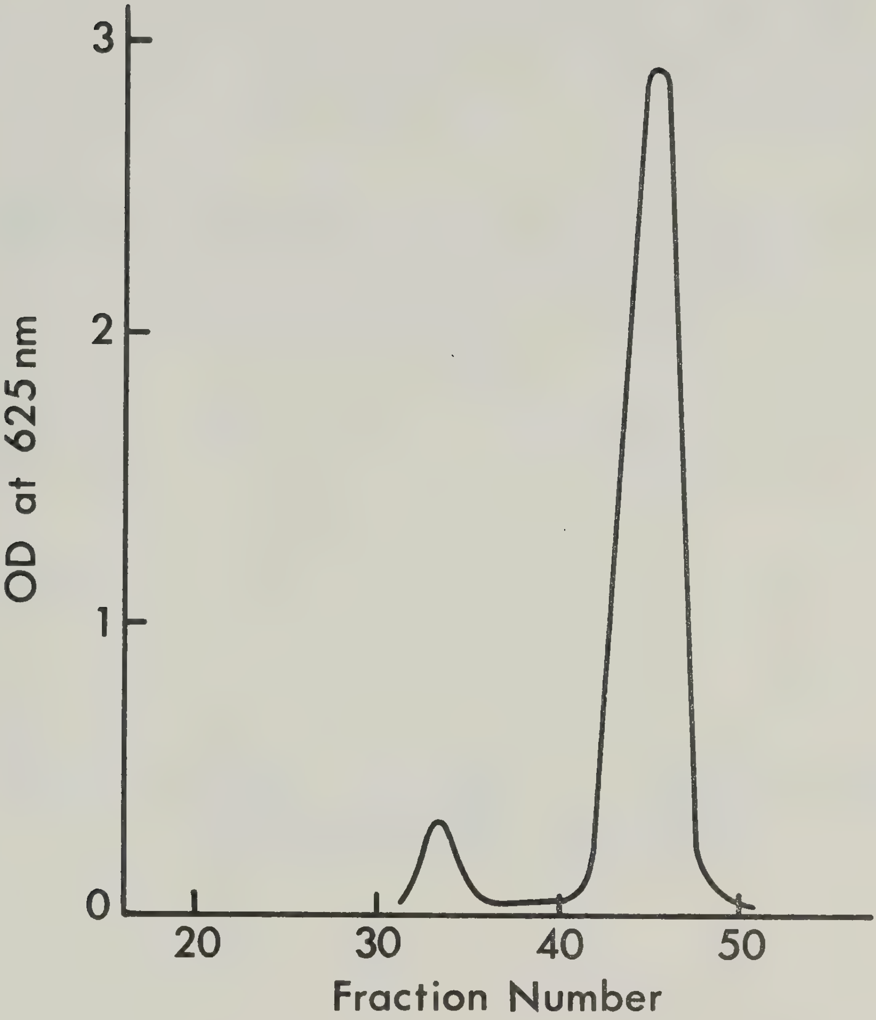

Fig. 15. Fractionation of spores of B. subtilis 8057 by density gradient centrifugation in renografin.

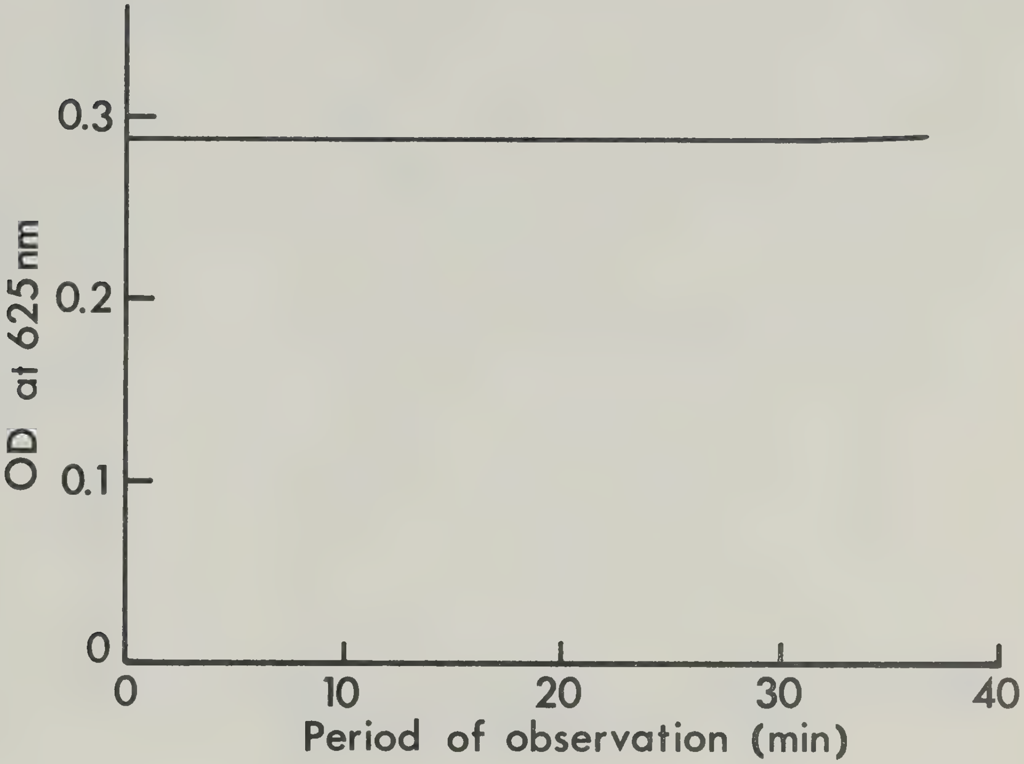

Fig. 16. Changes in O.D. occurring during incubation of light spores in Trypticase Soy Broth at 37°.

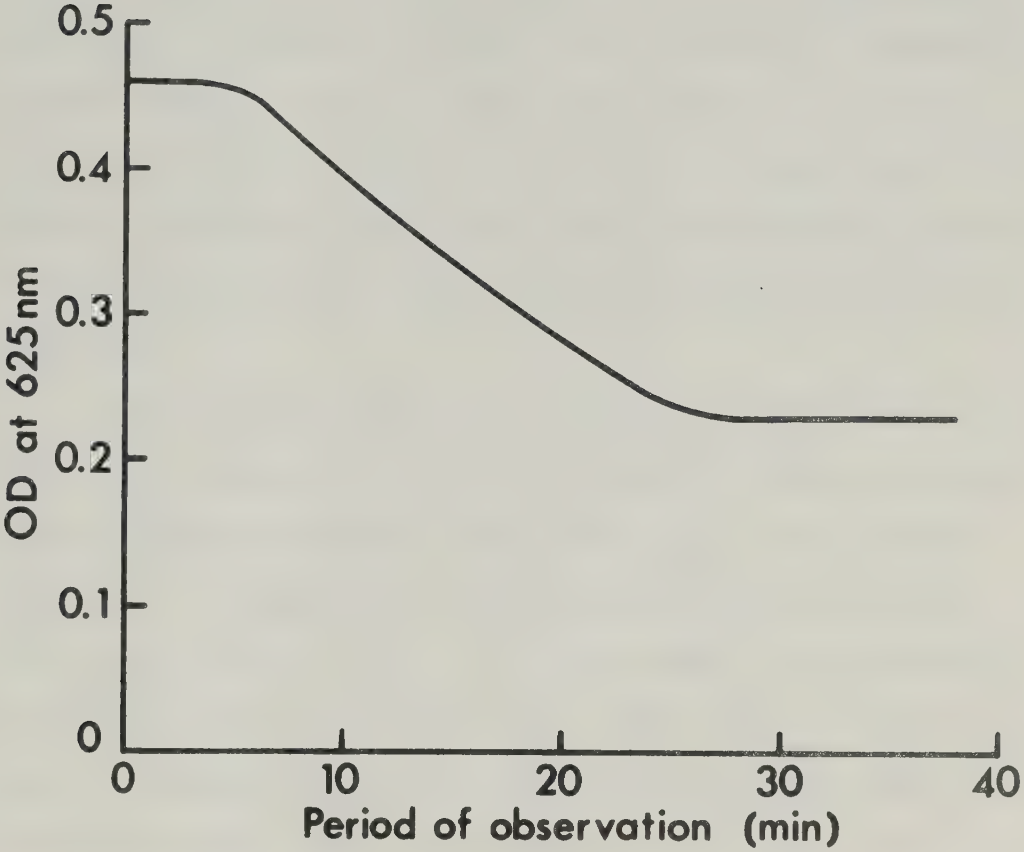

Fig. 17. Changes in O.D. occurring during incubation of heavy spores in Trypticase Soy Broth at 37°.

decreases typical of germinating spores. Conversely, the lighter bands showed no change in O.D. during the first hour of incubation in nutrient media: but later a slight increase in O.D. was observed. Microscopic examination showed these fractions to contain considerable numbers of vegetative cells. The lighter band, therefore, consisted of fully germinated spores rather than spores merely incapable of germination.

As a further test of this hypothesis, stock suspensions of spores were incubated in TSB at 37° for 30 min to induce germination without permitting outgrowth of vegetative cells. The incubated spores were then separated on density gradients of both sucrose (Fig. 18) and renografin (Fig. 19). It is obvious that incubation considerably alters the distribution of spores in the light and heavy bands. Microscopic examination of these bands showed no marked difference from the bands of the stock spore suspension. Therefore an effective separation of germinated from ungerminated spores had been achieved.

D. The Influence of Incubation Temperature on the Outgrowth of Bacillus subtilis Spores

The results obtained in section B suggest that the influence of incubation temperature on the recovery of heat-treated spores occurs at some stage other than germination. As the only other stage during recovery is the outgrowth of the germinated spores into vegetative cells and thus into visible colonies in nutrient media, it would seem that it is this stage at which the influence of incubation temperature is

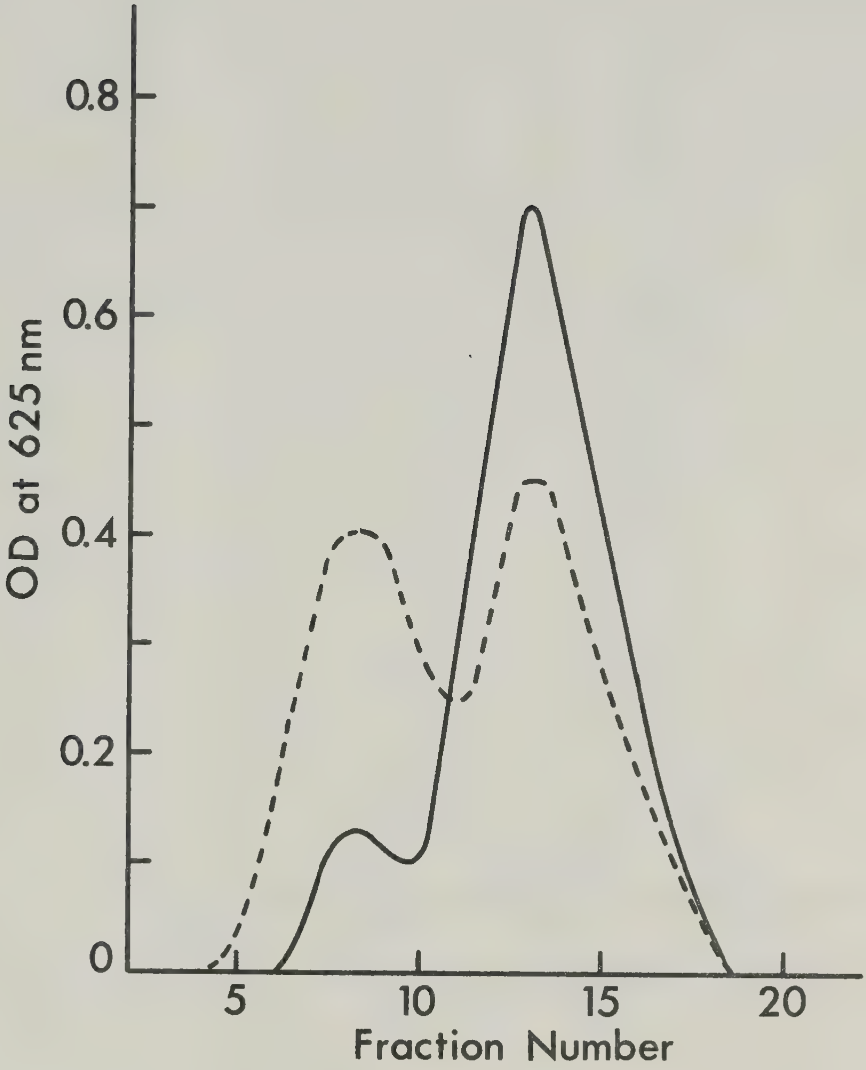

Fig. 18. The effect of incubation in Trypticase Soy Broth for 30 min at 37° on spores of B. subtilis 8057 fractionated by density gradient centrifugation in sucrose: continuous line, before; broken line, after.

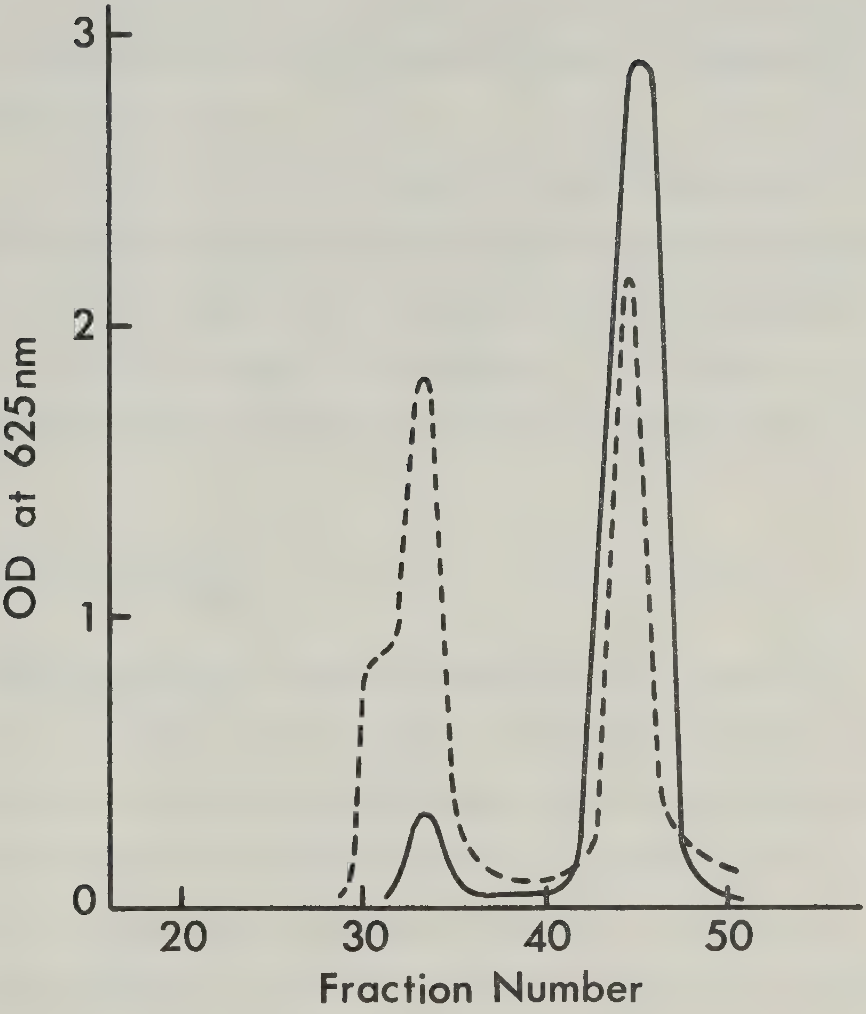

Fig. 19. The effect of incubation in Trypticase Soy Broth for 30 min at 37° on spores of B. subtilis 8057 fractionated by density gradient centrifugation in renografin: continuous line, before; broken line, after.

important. This would seem likely since outgrowth is a much more complex process than germination being principally biosynthetic rather than degradative.

In this study spores subjected to heat treatments of 0, 20, 40 and 60 min at 95° were allowed to germinate in nutrient media for 30 min and the germinated spores separated from the ungerminated fraction by density gradient centrifugation. Colony counts at different temperatures were then carried out on the germinated fraction.

1. Materials and Methods

a) Spore suspension. The spore suspension was prepared as described in section A. Because a very concentrated suspension was required the final working suspension was obtained by mixing the stock suspension with an equal volume of sterile 2 M phosphate buffer at pH 7. This gave a concentration yielding a SPC of c. 10^9 colonies/ml.

b) Heat treatment. The spore suspension was heated for different times at 95° in the manner described in section A.

c) Preparation of germinated, heat-treated spores. Two ml amounts of the heat-treated spore suspension were added to 100 ml of the sterile germination medium (see section B) at 37° in a sterile 250 ml conical flask. After incubation at 37° for 30 min the spores were centrifuged for 20 min at 10,000 rpm (12,000 g). After centrifugation the pellet formed was suspended in the minimum amount of water which would allow the suspension to be taken up in a Pasteur pipette. This suspension was then separated into germinated and ungerminated spores by density

gradient centrifugation as described in section C.

The band containing the ungerminated spores was carefully removed by means of a Pasteur pipette with the point bent at right angles to simplify removal. The suspension of germinated spores in renografin was diluted with distilled water, centrifuged at 10,000 rpm and the pellet formed was then taken up in 1 ml of distilled water and inoculated into 9 ml of quarter strength Ringer's solution. This was considered to be a 10^{-1} dilution of the suspension of germinated heat-treated spores.

d) Colony counts. Colony counts on the germinated spores were carried out in roll tubes in the temperature gradient incubator as described in section A. In this case however, because of the difficulty in anticipating the counts, serial dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} were incubated. Since the incubator contains only 6 holes at any one temperature, two adjacent rows were used for each count; one row containing duplicate tubes of the three lowest dilutions and the other row duplicate tubes of the higher dilutions. The temperature of the row giving a count of between 30 and 300 colonies/tube was taken as the temperature of incubation.

2. Results

Fig. 20 shows the effect of incubation temperature on the outgrowth of germinated spores of *B. subtilis* heated at 95° for 0, 20, 40 and 60 min. Because of the extensive manipulation of the cells before counting little can be read into the quantitative differences between treatments. This does not affect the validity of the relative

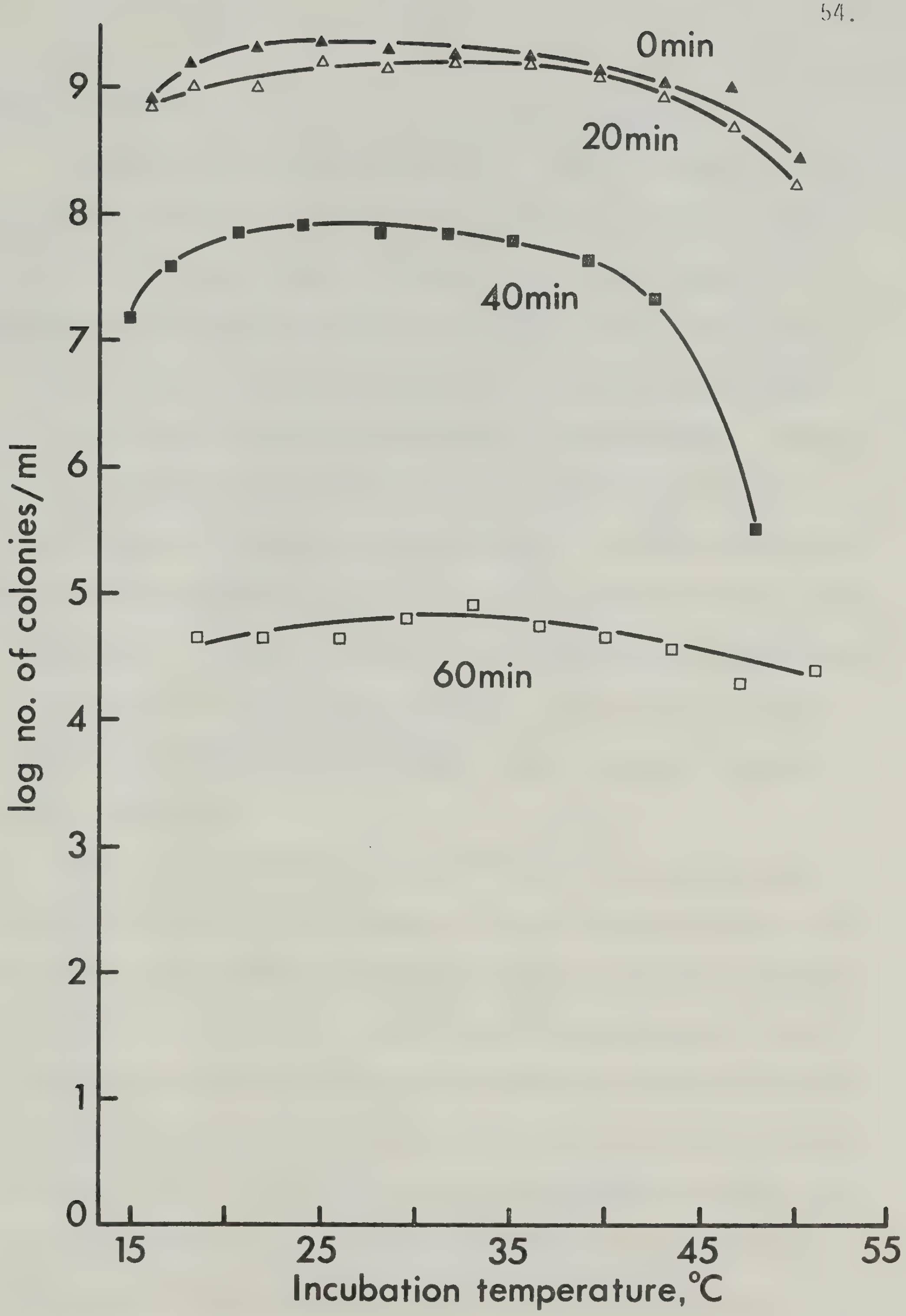

Fig. 20. The effect of incubation temperature on the outgrowth of _B. subtilis_ 8057 spores after treatment at 95° for different times and incubation at 37° for 30 min.

outgrowth measurements at different incubation temperatures.

The spores given the 0 and 20 min treatments show a similar extent of outgrowth over the range of temperatures tested while those treated for 40 min show a marked reduction in outgrowth above c. 42°. This agrees with the results obtained with ungerminated spores (Figs. 7 and 8) suggesting that it is the outgrowth of the heat-treated spore which is sensitive to incubation temperatures during recovery. However outgrowth of spores treated for 60 min at 95° showed less dependency on the temperature of incubation, the difference in counts between the highest incubation temperature used (53°) and the "optimum" being only half a log cycle. This does not agree with the above theory which would expect the outgrowth of spores given the most severe treatment to be more temperature dependent during recovery than the spores subjected to the other treatments.

During the separation of the germinated from ungerminated spores by density gradient centrifugation it was observed that, as the treatment became more severe, the number of spores in the germinated fraction decreased. The loss of viability during heating could cause this. Furthermore, as it has been shown in section B that heat-treated spores germinated more slowly than untreated spores, there existed the possibility that the 30 min period given in the germination medium may not have been sufficient to allow the germination of the majority of the viable spores. Accordingly, this part of the experiment was repeated allowing 60 min instead of 30 min (after heat-treatment at 95° for 60 min) for spores to germinate in the germination medium before centrifugation and separation of the germinated from the ungerminated

spores was effected.

These results are shown in Fig. 21 with the results obtained for spores given the same treatment but allowed to germinate for only 30 min. It can be seen that the spores allowed to germinate for the longer period show a greater sensitivity to temperature during outgrowth suggesting that the results obtained for the 60 min treatment in Fig. 20 are due to inadequate germination.

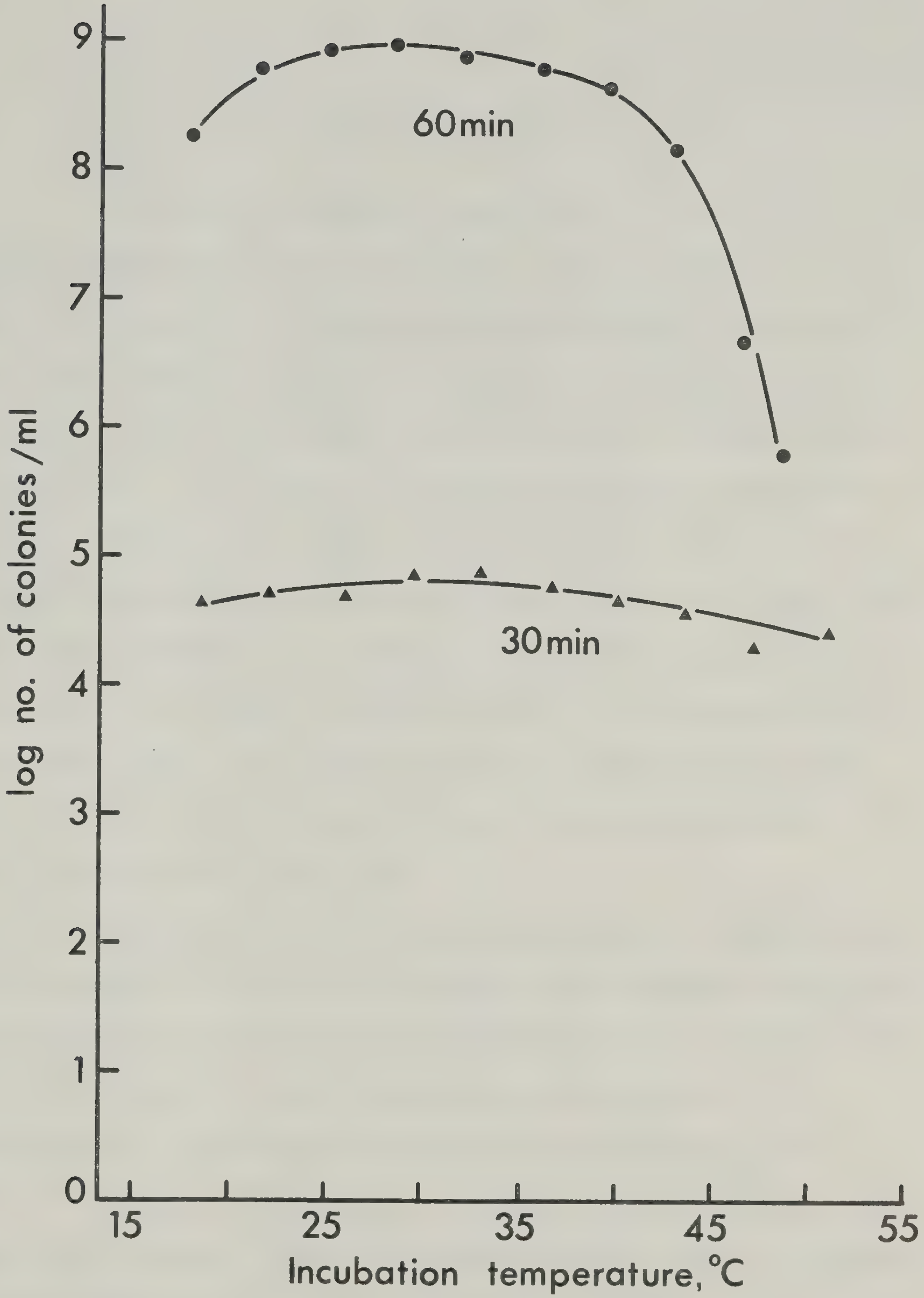

Fig. 21. The effect of incubation temperature on the outgrowth of B. subtilis 8057 spores after treatment at 95° for 60 min and incubation at 37° for different times.

III. DISCUSSION

The effect of incubation temperature on the recovery of *B. subtilis* spores heated at 95° for varying times was shown in Figs. 7 and 8 to increase with the duration of heat-treatment. Untreated spores gave a similar recovery with incubation temperatures from 16 - 50° whereas spores heat-treated for 70 min showed an optimum recovery in the range 24 - 32°. Both PCA and BHIA recovery media gave similar results. Edwards *et al*. (1965) found that severely heated spores of *B. subtilis* gave greater recovery at 32° than at 45°; this agrees with the results presented here. However, with untreated spores they found the converse to be true. No such difference in recovery was found in the present study. This might be because the unheated spores used in the present work were less affected by incubation temperature than those used by Edwards *et al*. (1965).

Although as stated earlier the composition of recovery media has a considerable effect on the recovery of heat damaged spores, it is not altogether surprising that PCA and BHIA gave similar results since both of these media are nutritionally relatively complex. In retrospect it is realized that more information might have been obtained had media differing more in composition been used (e.g., PCA and a minimal medium). Most workers agree that the recovery requirements of heat damaged cells are greater than those of undamaged cells: the present work demonstrates that with *B. subtilis* 8057, this also applies to the temperature of incubation during recovery.

Heat-treatment at 105° (Fig. 9) showed a similar temperature dependency which increased with the extent of treatment as did the treatment at 95° (Figs. 7 and 8). The temperature sensitivity of spores heated at 105° for 10 min was more extreme than in any treatment at 95° although this may not be an important difference. The results therefore suggest that treatments at the higher temperatures commonly used in processing would yield surviving spores which were similarly affected by temperature of incubation.

Since the dependency on incubation temperature during recovery increases with the severity of heat-treatment, it is interesting to speculate as to what would happen if the heat damage were more severe than that experimented with in this work. If the trend were to follow the same pattern, the dependency would become even greater and the incubation temperature would then have a most dramatic effect on the recovery of heat-treated cells.

It would thus have been desirable to carry out treatments at 115°, a temperature commonly used in food processing, however treatment at this temperature reduced the number of spores so rapidly that within 4 min there were no survivors. Therefore to do this would have required a capability for spore production for such experiments which was beyond the capacity of this laboratory.

Although incubation at c. 30° has been shown to give optimum recovery for heated _B. subtilis_ 8057 spores this is not the optimum recovery temperature for all industrially important organisms (e.g., _B. stearothermophilus_ spores heated for various times at 115° gave optimum recovery at 45 - 50° [Cook and Gilbert, 1968]). It

therefore seems unlikely that there is an ideal incubation temperature for studies on the recovery of heterogeneous spore populations as would be desirable in industrial processes. The present findings are of importance however since they demonstrate the necessity of carefully selecting the temperature or temperatures of incubation during thermal death studies and in carrying out quality control tests on heat processed foodstuffs. Perhaps incubation at different temperatures over a range should become routine.

The temperature gradient incubator designed for this study proved to be most useful and combined with the roll tubes allowed the enumeration of viable organisms over a wide range of temperatures. Other incubators of this nature have only been of use in the measurement of growth curves in broth cultures or maximum and minimum growth temperatures and it is believed that this is the first account of a temperature gradient incubator which permits colony counts to be made over its range. This should prove useful for a wide variety of studies.

In section B (Fig. 10) the optimum germination temperature of unheated spores agrees well with that obtained by Thorley and Wolf (1961) who found the optimum germination temperature for spores of B. subtilis in L-alanine to be 41°. This seems to have no relationship to the optimum temperature for growth of this organism or to the optimum recovery temperature of heat-damaged cells which are both in the region of 26°.

The incubation requirements for spore recovery were shown in Figs. 7, 8 and 9 to become more exacting with the severity of heat-

treatment. Fig. 11 shows that spores heated for 20 min at 95° can germinate at temperatures higher than unheated spores (Fig. 10). This might be due to heat activation. It was also demonstrated that spores heated at 95° for 20 min show some ability to germinate at temperatures which do not permit recovery. It therefore seems unlikely that any temperature dependency during recovery is caused by restrictions on germination. This is supported by the fact that Campbell (1957) and others have shown that spores can germinate in conditions unsuitable for outgrowth.

The studies on the rate of germination of spores at different temperatures using loss of heat-resistance as the criterion of germination (Figs. 12 and 13) showed that heat-treated spores germinated more slowly at 30° than unheated spores. The difficulties encountered during this study due to the very rapid initial rate of germination at 30° and the formation of fresh spores during the experimental run have been avoided by other workers, e.g., Thorley and Wolf (1961) who studied germination rates in media unsuitable for outgrowth. However, since the germination medium has been shown to affect the rate of germination it was decided that, to allow comparisons with previous results, the germination medium described should be used.

The use of density gradient centrifugation for the separation of germinated from ungerminated spores should prove to be of interest to investigators studying spore germination. Much of the existing work has been complicated by the difficulty in distinguishing between germination and outgrowth of already germinated spores. The method

reported herein should permit the preparation and purification of homogenous ungerminated and germinated spores, permitting the thorough investigation of parameters intrinsic to the steps in the germination process. Other workers have already speculated on differences in slow and fast segments of spores banded in renografin (Tamir and Gilvarg, 1966). Conceivably these might correspond to stages in germination.

In section C the effect of different incubation temperatures was studied on spores which had been heat-treated prior to germination. The results suggest that the germinated spores obtained by extensive germination of the suspension show a temperature dependency during outgrowth similar to that shown during the recovery of the ungerminated spores. It was also found that the optimum colony counts from outgrowth of the germinated, heat-treated spores were obtained in the range of incubation temperatures giving maximum recovery of ungerminated spores subjected to similar heat-treatments as shown in section A.

It thus seems likely that the temperature sensitivity during recovery described in section A is indeed caused by the limitations on outgrowth resulting from heat damage.

Heat-damaged spores allowed to germinate for 30 min show less temperature sensitivity during outgrowth than spores which have been allowed to germinate for 60 min (Fig. 21). Since in section B it was shown that on incubation at 30° damaged spores germinate more slowly than undamaged spores, 30 min incubation in the germination medium would permit the germination of only the least damaged cells of the population. The results obtained here therefore show, as would be

expected, that in the suspension the spores which have suffered least damage are least affected by incubation temperature during outgrowth. Hence, DGC of a heat-treated population after germination for different times should allow the separation of germinated spores into different fractions corresponding to the severity of damage the spores have suffered. Although the quantitative aspects of spore destruction have been studied extensively, little attention has been paid to the qualitative aspects and these findings may be of interest to workers in this area of research.

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APPENDIX A

COMPUTER DATA

1.

```
FORTRAN IV G COMPILER(21)       MAIN            02-20-73       18:50.06       PAGE 0001

            C
            C     TWO DIMENSIONAL STEADY STATE HEAT CONDUCTION
            C
            C
            C        THIS PROGRAM USES AN ITERATIVE PROCEDURE TO CALCULATE
            C     TEMPERATURE DURING STEADY STATE HEAT CONDUCTION IN TWO DIMENSIONS.
            C     THE SOLID IS REPRESENTED BY A NETWORK OF POINTS ON A SQUARE GRID
            C     OF UP TO 40 UNITS BY 80 UNITS. IN THE INPUT H AND C REPRESENT
            C     POINTS ON THE HOT AND COLD EDGES WITH TEMPERATURES TH AND TC,
            C     AND W AND K REPRESENT POINTS ON UNINSULATED EDGES WARMED AND
            C     COOLED BY SURROUNDINGS AT TEMPERATURES TSW AND TSK, RESPECTIVELY.
            C     ANY OTHER SYMBOL (E.G. A PERIOD (.) ) MAY BE USED TO REPRESENT
            C     OTHER POINTS IN THE SOLID, INCLUDING POINTS ON INSULATED EDGES.
            C     THE SOLID MUST BE SURROUNDED BY  BLANKS IN THE INPUT.
            C        THE NUMBER OF GRID INTERVALS FROM EACH POINT TO THE HOT, WARM,
            C     COOL AND COLD EDGES IS CALCULATED, AND THE INITIAL TEMPERATURES
            C     FOR THE CALCULATION ARE THEN CALCULATED USING A WEIGHTED AVERAGE
            C     OF THE TEMPERATURES AT THE NEAREST TWO HEATED OR COOLED EDGES.
            C        THE CALCULATION STOPS WHEN THE ROOT MEAN SQUARE RESIDUAL
            C     ERROR IS LESS THAN (TH-TC)/KSTOP. THE TEMPERATURES AND RESIDUAL
            C     ERRORS ARE THEN PRINTED.
            C        IMAX AND JMAX ARE THE NUMBER OF COLUMNS AND ROWS, RESPECTIVELY,
            C     IN THE INPUT ARRAY WHICH DESCRIBES THE SOLID, INCLUDING THE
            C     SURROUNDING BLANKS.
            C        BIW AND BIK ARE THE BIOT NUMBERS FOR HEAT TRANSFER AT THE
            C     HEATED AND COOLED EDGES OF THE SOLID. THE BIOT NUMBER  BI  IS
            C     GIVEN BY THE EQUATION
            C                           BI = H*D/K,
            C     WHERE  H IS THE HEAT TRANSFER COEFFICIENT, D IS THE DISTANCE
            C     BETWEEN ADJACENT POINTS ON THE SOLID, AND K IS THE THERMAL
            C     CONDUCTIVITY OF THE SOLID.
            C
            C
            C                THE PROGRAM
            C
            C     INITIALIZE VARIABLES AND DIMENSION ARRAYS
            C
0001              DIMENSION T(43,83)
0002              INTEGER*2  L(43,83),M(43,83),N(43,83),BLANK/' '/,H/'H'/,W/'W'/,C/'
                 CC'/,NL(86),K/'K'/
0003              INTEGER*4 DK(43,83),DW(43,83),DC(43,83),DH(43,83),DTOT,REP
0004              INTEGER*4 DC1,DC2,DC3,DC4,DK1,DK2,DK3,DK4,DW1,DW2,DW3,DW4,DH1,DH2,
                 CDH3,DH4
0005              INTEGER*4 DKIJ,DWIJ,DCIJ,DHIJ,DMAX
0006              REAL*4 BLANCK/'    '/,R(43,83)
0007              REAL*8 A/',A4,2X'/,B/',F6.1'/,FMT(25)/'(T2',',I2',',T4',21*',F6.1'
                 C,')'/,BR/',F6.3'/
            C
            C     READ  SHAPE OF SOLID, TEMPERATURES AND HEAT TRANSFER DATA.
            C
0008           10 READ(5,900,END=999)IMAX,JMAX,KSTOP
0009              READ(5,901)TH,TC,TSW,TSK,BIW,BIK
0010              DO 15 J=1,83
0011              DO 15 I=1,43
0012              M(I,J)=0
```

```
 0013          15 L(I,J)=0
 0014             TS=0
 0015             DO 19 J=1,JMAX
 0016          19 READ(5,902)(M(I,J),I=1,IMAX)
            C
            C     DETERMINE VALUES OF CALCULATION PARAMETERS
            C
 0017             NP=0
 0018             DO 21 J=1,JMAX
 0019             DO 21 I=1,IMAX
 0020             IF(M(I,J).NE.BLANK) GO TO 20
 0021             T(I,J)=0
 0022             GO TO 21
 0023          20 N(I,J)=1
 0024             L(I,J)=2
 0025             NP=NP+1
 0026          21 CONTINUE
 0027             MAXJ=JMAX-1
 0028             MAXI=IMAX-1
 0029             DO 27 J=2,MAXJ
 0030             DO 27 I=2,MAXI
 0031             IF(L(I,J).EQ.0) GO TO 27
 0032             L8=L(I,J+1)*L(I+1,J+1)*L(I+1,J)*L(I+1,J-1)*L(I,J-1)*L(I-1,J-1)*L(I
                C-1 ,J)*L(I-1,J+1)
 0033             IF(L8.NE.0) GO TO 22
 0034             L(I,J)=1
 0035             N(I,J)=2
 0036          22 IF(M(I,J).NE.K) GO TO 23
 0037             N(I,J)=3
 0038             GO TO 27
 0039          23 IF(M(I,J).NE.W) GO TO 24
 0040             N(I,J)=4
 0041             GO TO 27
 0042          24 IF(M(I,J).NE.C) GO TO 25
 0043             N(I,J)=5
 0044             T(I,J)=TC
 0045             NP=NP-1
 0046             GO TO 27
 0047          25 IF(M(I,J).NE.H) GO TO 27
 0048             N(I,J)=6
 0049             T(I,J)=TH
 0050             NP=NP-1
 0051          27 CONTINUE
            C
            C     PRINT OUT SHAPE OF SOLID AND TABLES OF CALCULATION PARAMETERS
            C
 0052             WRITE(6,903)
 0053             DO 30 J=1,JMAX
 0054          30 WRITE(6,904)(M(I,J),I=1,IMAX)
 0055             WRITE(6,905)
 0056             WRITE(6,906)
 0057             DO 32 J=1,JMAX
 0058             DO 31 I=1,43
 0059             NI(I)=N(I,J)
 0060          31 NL(I+43)=L(I,J)
```

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FORTRAN IV G COMPILER(21)       MAIN            02-20-73      18:50.06      PAGE 0003

                    32 WRITE(6,907)(NL(I),I=1,86)
                       WRITE(6,908)
                 C
                 C     CALCULATE INITIAL TEMPERATURE DISTRIBUTION
                 C
                       DO 200 I=1,IMAX
                       DO 200 J=1,JMAX
                       DK(I,J)=99999
                       DW(I,J)=99999
                       DC(I,J)=99999
                       DH(I,J)=99999
                       IF(N(I,J).EQ.3) DK(I,J)=1/BIK
                       IF(N(I,J).EQ.4) DW(I,J)=1/BIW
                       IF(N(I,J).EQ.5) DC(I,J)=0
                       IF(N(I,J).EQ.6) DH(I,J)=0
                   200 CONTINUE
                       KI=0
                       KJ=0
                   205 KI=(KI-KJ)*(KI-KJ)
                       KJ=1-KJ
                       REP=0
                       DO 215 JR=2,MAXJ
                       DO 215 IR=2,MAXI
                       I=IR*KI+(IMAX+1-IR)*(1-KI)
                       J=JR*KJ+(JMAX+1-JR)*(1-KJ)
                       IF(N(I,J).EQ.0) GO TO 215
                       DTOT=DC(I,J)+DH(I,J)+DK(I,J)+DW(I,J)
                       DC1=DC(I,J+1)
                       DC2=DC(I+1,J)
                       DC3=DC(I,J-1)
                       DC4=DC(I-1,J)
                       DK1=DK(I,J+1)
                       DK2=DK(I+1,J)
                       DK3=DK(I,J-1)
                       DK4=DK(I-1,J)
                       DW1=DW(I,J+1)
                       DW2=DW(I+1,J)
                       DH3=DH(I,J-1)
                       DH4=DH(I-1,J)
                       DH1=DH(I,J+1)
                       DH2=DH(I+1,J)
                       DW3=DW(I,J-1)
                       DW4=DW(I-1,J)
                       IF(N(I,J).EQ.3) GO TO 206
                       DK(I,J)=MINO(DK1,DK2,DK3,DK4)+1
                   206 IF(N(I,J).EQ.4) GO TO 207
                       DW(I,J)=MINO(DW1,DW2,DW3,DW4)+1
                   207 IF(N(I,J).EQ.5) GO TO 208
                       DC(I,J)=MINO(DC1,DC2,DC3,DC4)+1
                   208 IF(N(I,J).EQ.6) GO TO 209
                       DH(I,J)=MINO(DH1,DH2,DH3,DH4)+1
                   209 REP=REP+DK(I,J)+DW(I,J)+DC(I,J)+DH(I,J)-DTOT
                   215 CONTINUE
                       IF(REP.NE.0) GO TO 205
                       WRITE(6,915)
```

```
      DO 220 J=1,JMAX
  220 WRITE(6,916) (DK(I,J),I=1,IMAX)
      WRITE(6,917)
      DO 221 J=1,JMAX
  221 WRITE(6,916) (DW(I,J),I=1,IMAX)
      WRITE(6,918)
      DO 222 J=1,JMAX
  222 WRITE(6,916) (DC(I,J),I=1,IMAX)
      WRITE(6,919)
      DO 223 J=1,JMAX
  223 WRITE(6,916) (DH(I,J),I=1,IMAX)
      DMAX=0
  219 DO 230 I=2,MAXI
      DO 230 J=2,MAXJ
      NIJ1=N(I,J)+1
      GO TO (230,226,226,226,226,224,225),NIJ1
  224 T(I,J)=TC
      GO TO 230
  225 T(I,J)=TH
      GO TO 230
  226 DKIJ=DK(I,J)
      DWIJ=DW(I,J)
      DCIJ=DC(I,J)
      DHIJ=DH(I,J)
      IF(DKIJ.LT.99999) DKIJ=DKIJ-1/BIK
      IF(DWIJ.LT.99999) DWIJ=DWIJ-1/BIW
      DO 260 K=1,2
      IF(DWIJ.NE.MAX0(DHIJ,DCIJ,DWIJ,DKIJ)) GO TO 251
      DWIJ=-99999
      GO TO 260
  251 IF(DKIJ.NE.MAX0(DHIJ,DCIJ,DWIJ,DKIJ)) GO TO 252
      DKIJ=-99999
      GO TO 260
  252 IF(DHIJ.NE.MAX0(DHIJ,DCIJ,DWIJ,DKIJ)) GO TO 253
      DHIJ=-99999
      GO TO 260
  253 DCIJ=-99999
  260 CONTINUE
      T(I,J)=(TH/DHIJ+TC/DCIJ+TSW*BIW/(BIW*DWIJ+1)+TSK*BIK/(BIK*DKIJ+1))
     C/(1.0/DHIJ+1.0/DCIJ+BIW/(BIW*DWIJ+1)+BIK/(BIK*DKIJ+1))
      DMAX=MAX0(DMAX,DKIJ,DWIJ,DCIJ,DHIJ)
  230 CONTINUE
C
C     PRINT TABLE OF INITIAL TEMPERATURES
C
      WRITE(6,920) (I,I=2,22)
      INIT=0
      GO TO 117
  231 INIT=1
C
C     CALCULATE TEMPERATURES
C
      WRITE(6,921)
      NIT=0
      KI=0
```

```
      KJ=0
   39 NITR=0
   40 KI=(KI-KJ)*(KI-KJ)
      KJ=1-KJ
      NIT=NIT+1
      NITR=NITR+1
      DO 51 JR=2,MAXJ
      DO 51 IR=2,MAXI
      I=IR*KI+(IMAX+1-IR)*(1-KI)
      J=JR*KJ+(JMAX+1-JR)*(1-KJ)
      TIJ=T(I,J)
      NIJ=N(I,J)
      GO TO(45,45,45,45),NIJ
      GO TO 51
   45 T1=T(I,J+1)
      T2=T(I+1,J)
      T3=T(I,J-1)
      T4=T(I-1,J)
      BQ=0
      GO TO (49,48,46,47),NIJ
   46 BQ=BIK
      TS=TSK
      GO TO 48
   47 BQ=BIW
      TS=TSW
   48 L1=L(I,J+1)
      L2=L(I+1,J)
      L3=L(I,J-1)
      L4=L(I-1,J)
      T(I,J)=(L1*T1+L2*T2+L3*T3+L4*T4+2*BQ*TS)/(L1+L2+L3+L4+2*BQ)
      T(I,J)=T(I,J)+(T(I,J)-TIJ)*0.5
      GO TO 51
   49 T(I,J)=(T1+T2+T3+T4)/4
      T(I,J)=T(I,J)+(T(I,J)-TIJ)*0.5
   51 CONTINUE
C
C     CALCULATE RESIDUALS AND TEST FOR END OF CALCULATION
C
      IF(NITR.LT.5 ) GO TO 40
      IF(NIT.LE.3*DMAX) GO TO 40
      SRESQ=0
      DO 80 I=2,MAXI
      DO 80 J=2,MAXJ
      BQ=0
      NIJ1=N(I,J)+1
      GO TO (79,73,72,71,70,79,79),NIJ1
   70 BQ=BIW
      TS=TSW
      GO TO 72
   71 BQ=BIK
      TS=TSK
   72 L1=L(I,J+1)
      L2=L(I+1,J)
      L3=L(I,J-1)
      L4=L(I-1,J)
```

```
            73 T0=T(I,J)
               T1=T(I,J+1)
               T2=T(I+1,J)
               T3=T(I,J-1)
               T4=T(I-1,J)
               GO TO (80,78,77,77,77),NIJ1
            77 R(I,J)=(L1*(T1-T0)+L2*(T2-T0)+L3*(T3-T0)+L4*(T4-T0))/2+BQ*(TS-T0)
               GO TO 80
            78 R(I,J)=T1+T2+T3+T4-4*T0
               GO TO 80
            79 R(I,J)=0
            80 SRESQ=SRESQ+R(I,J)*R(I,J)
               RMSQR=(SRESQ/NP)**0.5
               WRITE(6,922)NIT,RMSQR
               IF(RMSQR.GT.(TH-TC)/KSTOP) GO TO 39
      C
      C        PRINT TABLE(S) OF TEMPERATURES
      C
               WRITE(6,909) (I,I=2,22)
           117 DO 121 J=2,MAXJ
               DO 118 IFMT=4,24
           118 FMT(IFMT)=B
               DO 119 I=2,22
               IF(N(I,J).NE.0) GO TO 119
               IFMT=I+2
               T(I,J)=BLANCK
               FMT(IFMT)=A
           119 CONTINUE
               WRITE(6,FMT)J,(T(I,J),I=2,22)
               DO 120 I=2,22
               IF(N(I,J).EQ.0)T(I,J)=0
           120 CONTINUE
           121 CONTINUE
               IF(IMAX.LT.24) GO TO 131
               IF(INIT.EQ.0) WRITE(6,923)(I,I=22,42)
               IF(INIT.EQ.1) WRITE(6,910)(I,I=22,42)
               DO 131 J=2,MAXJ
               DO 128 IFMT=4,24
           128 FMT(IFMT)=B
               DO 129 I=22,42
               IF(N(I,J).NE.0) GO TO 129
               IFMT=I-18
               T(I,J)=BLANCK
               FMT(IFMT)=A
           129 CONTINUE
               WRITE(6,FMT) J,(T(I,J),I=22,MAXI)
               DO 130 I=22,42
               IF(N(I,J).EQ.0)T(I,J)=0
           130 CONTINUE
           131 CONTINUE
               IF(INIT.EQ.0) GO TO 231
      C
      C        PRINT OUT TABLE(S) OF RESIDUALS
      C
               WRITE(6,911)NIT,NP,RMSQR
```

```
FORTRAN IV G COMPILER(21)       MAIN            02-20-73     18:50.06      PAGE 0007

0262              WRITE(6,912) (I,I=2,22)
0263              DO 157 J=2,MAXJ
0264              DO 156 IFMT=4,24
0265          156 FMT(IFMT)=ER
0266              DO 149 I=2,22
0267              IF(N(I,J).NE.0) GO TO 149
0268              IFMT=I+2
0269              R(I,J)=BLANCK
0270              FMT(IFMT)=A
0271          149 CONTINUE
0272              WRITE(6,FMT) J,(R(I,J),I=2,22)
0273          157 CONTINUE
0274              IF(IMAX.LT.24) GO TO 170
0275              WRITE(6,913) (I,I=22,42)
0276              DO 161 J=2,JMAX
0277              DO 158 IFMT=4,24
0278          158 FMT(IFMT)=ER
0279              DO 159 I=22,42
0280              IF(N(I,J).NE.0) GO TO 159
0281              IFMT=I-18
0282              R(I,J)=BLANCK
0283              FMT(IFMT)=A
0284          159 CONTINUE
0285              WRITE(6,FMT) J,(R(I,J),I=22,MAXI)
0286          161 CONTINUE
0287          170 GO TO 10
            C
            C     FORMAT STATEMENTS
            C
0288          900 FORMAT(2I2,I6)
0289          901 FORMAT(4F5.1,2F7.5)
0290          902 FORMAT(43A1)
0291          903 FORMAT('1',T20,'TWO-DIMENSIONAL HEAT CONDUCTION'///)
0292          904 FORMAT(T20,43A1)
0293          905 FORMAT( //T20,'FIGURE 1. SHAPE OF THE SOLID.'//
                 K     T22,'SOLID             .'/
                 K     T22,'HOT EDGE          H'/
                 K     T22,'COLD EDGE         C'/
                 K     T22,'UNINSULATED EDGE'/T24,'(WARM)',T40,'W'/
                 K     T22,'UNINSULATED EDGE'/T24,'(COOL)',T40,'K')
0294          906 FORMAT('1',T20,'CALCULATION PARAMETERS'///)
0295          907 FORMAT(T20,43I1,T70,43I1)
0296          908 FORMAT(  /T30,'FIGURE 2. ARRAY N(I,J).',T80,'FIGURE 3. ARRAY L(I,J
                 K).'//T32,'SPACE             0',T82,'SPACE             0'/
                 K     T32,'INTERIOR OF SOLID 1',T82,'EDGE OF SOLID     1'/
                 K     T32,'INSULATED EDGE    2',T82,'INTERIOR OF SOLID 2'/
                 K     T32,'UNINSULATED EDGE'/T34,'(COOL)',T50,'3'/
                 K     T32,'UNINSULATED EDGE'/T34,'(WARM)',T50,'4'/
                 K     T32,'COLD EDGE         5'/
                 K     T32,'HOT EDGE          6')
0297          909 FORMAT('1',T10,'TABLE OF TEMPERATURES'///T4,'I'/'+',T3,21I6//T3,'J
                 C'/)
0298          910 FORMAT('1',T10,'TABLE OF TEMPERATURES (CONTINUED)'///T4,'I'/'+',T3
                 C,21I6//T3,'J'/)
0299          911 FORMAT(  //T30,'NUMBER OF ITERATIONS = ',I5,T60,'NUMBER OF POINTS
```

```
FORTRAN IV G COMPILER(21)       MAIN            02-20-73     18:50.06       PAGE 0008

                   K= ',I4/T30,'ROOT MEAN SQUARE RESIDUAL = ',F8.6)
 0300           912 FORMAT('1',T10,'TABLE OF RESIDUALS   '///T4,'I'/'+',T3,21I6//T3,'J
                   C'/)
 0301           913 FORMAT('1',T10,'TABLE OF RESIDUALS (CONTINUED)   '///T4,'I'/'+',T3
                   C,21I6//T3,'J'/)
 0302           914 FORMAT(T30,'OVERSHOOT = ',F5.1,' PERCENT')
 0303           915 FORMAT('1',T20,'TABLE OF DK(I,J)'//)
 0304           916 FORMAT(T2 ,43I3)
 0305           917 FORMAT('1',T20,'TABLE OF DW(I,J)'//)
 0306           918 FORMAT('1',T20,'TABLE OF DC(I,J)'//)
 0307           919 FORMAT('1',T20,'TABLE OF DH(I,J)'//)
 0308           920 FORMAT('1',T20,'TABLE OF INITIAL TEMPERATURES'///,T4,'I'/'+',T3,21
                   CI6//T3,'J'/)
 0309           921 FORMAT('1',T10,'NIT',T17,'RMSQR'/)
 0310           922 FORMAT(T10,I3,T16,F8.6)
 0311           923 FORMAT('1',T20,'TABLE OF INITIAL TEMPERATURES (CONTINUED)'///T4,'I
                   C'/'+',T3,21I6//T3,'J'/)
 0312           999 STOP
 0313               END
```

2. The Unjacketed Incubator

Note:

The constants 2.000900 and 0.000900 in input statement 0007 are 2 + R and R respectively, where R is given by the equation

$$R = \frac{\Delta x_b \,/\, k_b A_b}{\Delta x_w \,/\, k_w A_w + \Delta x_p \,/\, k_p A_p}$$

where subscripts p, w and b refer to the layer of polystyrene insulation, the layer of wood surrounding the insulation and the temperature gradient bar, respectively. The terms k, A and Δx are thermal conductivity, area normal to the direction of heat flow, and distance in the direction of heat flow, respectively.

```
FORTRAN IV G COMPILER(21)       MAIN            02-20-73      18:49.53      PAGE 0001

                 READ(5,87,END=999)TH,TC,TS,DTB
                 DIMENSION TB(21),        ERRB(21)
                 TB(1)=TH
                 NIT=0
              47 TB(2)=TH-DTB
                 DO 57 N=2,20
              57 TB(N+1)=2.000900*TB(N)-TB(N-1)-0.000900*TS
                 IF(ABS(TB(21)-TC).LT.DTB/100 ) GO TO 67
                 DTB=DTB*(TH-TC)/(TH-TB(21))
                 NIT=NIT+1
                 IF(NIT.LT.20) GO TO 47
              67 DTB=(TH-TB(21))/20
                 DO 68 N=1,21
              68 ERRB(N)=(N-1)*DTB+TB(N)-TH
                 WRITE(6,89)TH,TC,TS
                 DO 71 N=1,21
              71 WRITE(6,91)N,      TB(N),ERRB(N)
                 WRITE(6,92) NIT
              87 FORMAT(5F5.2)
              89 FORMAT('1',T20,'TEMPERATURES IN UNJACKETED TEMPERATURE GRADIENT IN
                CCUBATOR'///T25,'TEMPERATURE AT HOT END = ',F5.2/T25,'TEMPERATURE A
                CT COLD END = ',F5.2/T25,'AMBIENT TEMPERATURE = ',F5.2///T20,'BLOCK
                C',T27,'TEMPERATURE',T41,'ERROR IN BAR'/T20,'NUMBER',T29,'OF BAR',
                CT41,'TEMPERATURE'/T27,'(DEGREES C)',T41,'(DEGREES C)'/)
              91 FORMAT(T22,I2,T29,F7.4,T43,F7.4)
              92 FORMAT(//////T20,'NO OF ITERATIONS = ',I2)
             999 STOP
                 END
```

```
TEMPERATURES IN UNJACKETED TEMPERATURE GRADIENT INCUBATOR


     TEMPERATURE AT HOT END = 57.00
     TEMPERATURE AT COLD END = 24.00
     AMBIENT TEMPERATURE = 20.00


BLOCK  TEMPERATURE   ERROR IN BAR
NUMBER   OF BAR      TEMPERATURE
       (DEGREES C)   (DEGREES C)

   1     57.0000        0.0
   2     55.1383       -0.2116
   3     53.3081       -0.3916
   4     51.5078       -0.5416
   5     49.7359       -0.6634
   6     47.9907       -0.7584
   7     46.2706       -0.8283
   8     44.5741       -0.8746
   9     42.8597       -0.8988
  10     41.2459       -0.9025
  11     39.6112       -0.8871
  12     37.9940       -0.8540
  13     36.3930       -0.8048
  14     34.8068       -0.7409
  15     33.2338       -0.6637
  16     31.6727       -0.5747
  17     30.1220       -0.4751
  18     28.5805       -0.3665
  19     27.0466       -0.2502
  20     25.5190       -0.1276
  21     23.9964       -0.0000
```

3. The Jacketed Incubator

Note:

The constants 2.000993 and 0.000993 in input statement 0009 are $2 + R_1$ and R_1 respectively, and the constants 2.004095, 0.001899 and 0.002196 in input statement 0010 are $2 + R_2 + R_3$, R_2 and R_3 respectively. R_1, R_2 and R_3 are given by the following equations:

$$R_1 = \frac{k_{pi}A_{pi}}{\Delta x_{pi}} \bigg/ \frac{k_b A_b}{\Delta x_b}$$

$$R_2 = \frac{k_{pi}A_{pi}}{\Delta x_{pi}} \bigg/ \frac{k_j Aj}{\Delta x_j}$$

$$R_3 = \frac{\Delta x_j \,/\, k_j A_j}{\Delta x_{po} \,/\, k_{po} A_{po} + \Delta x_w \,/\, k_w A_w}$$

The subscripts b, pi, po, j, and w refer to the gradient bar, the inner and outer layers of polystyrene, the jacket and the outer layer of plywood. The terms R, A and Δx are thermal conductivity, area normal to the direction of heat flow, and distance in the direction of heat flow, respectively.

```
FORTRAN IV G COMPILER(21)        MAIN             02-20-73      18:49.36      PAGE 0001

                  READ(5,87,END=999)TH,TC,TS,DTB,DTJ
                  DIMENSION TB(21),TJ(21),ERRB(21)
                  TB(1)=TH
                  TJ(1)=TH
                  NIT=0
               47 TB(2)=TH-DTB
                  TJ(2)=1.002196*TH-DTJ-0.002196*TS
                  DO 57 N=2,20
                  TB(N+1)=2.000993*TB(N)-TB(N-1)-0.000993*TJ(N)
               57 TJ(N+1)=2.004095*TJ(N)-TJ(N-1)-0.001899*TB(N)-0.002196*TS
                  IF(ABS(TB(21)-TJ(21)).LT.DTB/100 .AND.ABS(TB(21)-TC).LT.DTB/100 )G
                 KO TO 67
                  DTB=DTB*(TH-TC)/(TH-TB(21))
                  DTJ=DTJ*(TH-TC)/(TH-TJ(21))
                  NIT=NIT+1
                  IF(NIT.LT.20) GO TO 47
               67 DTB=(TH-TB(21))/20
                  DO 68 N=1,21
               68 ERRB(N)=(N-1)*DTB+TB(N)-TH
                  WRITE(6,89)TH,TC,TS
                  DO 71 N=1,21
               71 WRITE(6,91)N,TJ(N),TB(N),ERRB(N)
                  WRITE(6,92) NIT
               87 FORMAT(5F5.2)
               89 FORMAT('1',T20,'TEMPERATURES IN JACKETED TEMPERATURE GRADIENT INCU
                 KBATOR'///T25,'TEMPERATURE AT HOT END = ',F5.2/T25,'TEMPERATURE AT
                 KCOLD END = ',F5.2/T25,'AMBIENT TEMPERATURE = ',F5.2///T20,'BLOCK',
                 KT27,2('TEMPERATURE   '),T55,'ERROR IN BAR'/T20,'NUMBER',T28,'OF JA
                 KCKET',T43,'OF BAR',T55,'TEMPERATURE'/T27,'(DEGREES C)',T41,'(DEGRE
                 KES C)',T55,'(DEGREES C)'/)
               91 FORMAT(T22,I2,T29,F7.4,T43,F7.4,T57,F7.4)
               92 FORMAT(//////T20,'NO OF ITERATIONS = ',I2)
              999 STOP
                  END
```

```
TEMPERATURES IN JACKETED TEMPERATURE GRADIENT INCUBATOR


     TEMPERATURE AT HOT END = 57.00
     TEMPERATURE AT COLD END = 24.00
     AMBIENT TEMPERATURE = 20.00


BLOCK  TEMPERATURE   TEMPERATURE   ERROR IN BAR
NUMBER  OF JACKET      OF BAR      TEMPERATURE
       (DEGREES C)   (DEGREES C)   (DEGREES C)

   1      57.0000       57.0000        0.0
   2      54.8741       55.3372      -0.0129
   3      52.8239       53.6749      -0.0253
   4      50.8441       52.0133      -0.0369
   5      48.9297       50.3529      -0.0474
   6      47.0761       48.6939      -0.0565
   7      45.2788       47.0365      -0.0640
   8      43.5336       45.3808      -0.0698
   9      41.8365       43.7270      -0.0737
  10      40.1837       42.0750      -0.0758
  11      38.5716       40.4248      -0.0761
  12      36.9967       38.7764      -0.0745
  13      35.4557       37.1298      -0.0712
  14      33.9454       35.4849      -0.0663
  15      32.4628       33.8414      -0.0598
  16      31.0048       32.1992      -0.0520
  17      29.5687       30.5583      -0.0431
  18      28.1517       28.9183      -0.0332
  19      26.7511       27.2790      -0.0226
  20      25.3642       25.6402      -0.0114
  21      23.9886       24.0017       0.0
```

APPENDIX B

BACTERIOLOGICAL RESULTS

1. The effect of incubation temperature on the recovery in Plate Count Agar of *B. subtilis* 8057 spores after treatment at 95° for different times (see Fig. 7).

0 treatment		5 min treatment	
Converted average colony count/ml	incubation temperature	Converted average colony count/ml	incubation temperature
4.2×10^7	50.0	$<5 \times 10^4$	56.0
6.0×10^7	48.5	2.5×10^6	54.0
6.2×10^7	47.0	3.1×10^7	52.0
6.0×10^7	45.3	1.8×10^7	50.0
5.5×10^7	43.7	3.1×10^7	48.0
7.2×10^7	42.2	4.1×10^7	46.0
7.3×10^7	40.6	4.6×10^7	44.0
9.6×10^7	39.2	5.9×10^7	42.0
8.0×10^7	37.5	5.6×10^7	40.0
9.0×10^7	36.0	6.1×10^7	38.0
8.5×10^7	34.5	5.8×10^7	36.0
8.5×10^7	33	8.5×10^7	34.0
1.0×10^8	31.3	8.3×10^7	32.0
9.5×10^7	29.8	8.8×10^7	30.0
1.0×10^8	28.1	8.5×10^7	28.0
9.5×10^7	26.7	9.2×10^7	26.0
9.0×10^7	25.0	8.8×10^7	24.0
8.3×10^7	23.4	7.7×10^7	22.0
9.0×10^7	21.9	8.3×10^7	20.0
9.0×10^7	20.5	8.4×10^7	18.0
-	19.0	7.4×10^7	16.0

10 min treatment		15 min treatment	
Converted average colony count/ml	incubation temperature	Converted average colony count/ml	incubation temperature
$<5 \times 10^4$	56.0	<50	56.0
$<5 \times 10^4$	54.0	<50	54.0
$<5 \times 10^4$	52.0	<50	52.0
7.5×10^4	50.0	5.2×10^4	50.0
1.6×10^6	48.0	-	47.9
8.5×10^6	46.0	-	45.8
3.1×10^7	44.0	-	43.8
4.4×10^7	42.0	4.3×10^7	41.8
5.4×10^7	40.0	4.2×10^7	39.7
6.2×10^7	38.0	5.7×10^7	37.6
5.9×10^7	36.0	5.3×10^7	35.5
6.0×10^7	34.0	5.5×10^7	33.5
7.0×10^7	32.0	4.8×10^7	31.4
7.1×10^7	30.0	5.6×10^7	29.4
7.2×10^7	28.0	4.9×10^7	27.3
6.8×10^7	26.0	4.7×10^7	25.2
6.1×10^7	24.0	4.6×10^7	23.1
4.5×10^7	22.0	4.0×10^7	21.1
4.6×10^7	20.0	3.8×10^7	19.0
4.2×10^7	18.0	3.8×10^7	17.0
-	16.0	-	15.0

20 min treatment		25 min treatment	
Converted average colony count/ml	incubation temperature	Converted average colony count/ml	incubation temperature
<50	56.0	<5	56.0
<50	54.0	<5	54.0
<50	52.0	<5	52.0
<50	50.0	255	50.0
6.7×10^4	47.9	5.0×10^4	67.9
-	45.8	5.9×10^5	45.8
-	43.8	3.9×10^6	43.8
1.9×10^7	41.8	1.4×10^7	41.8
2.1×10^7	39.7	1.8×10^7	39.7
2.0×10^7	37.6	1.8×10^7	37.6
2.2×10^7	35.5	2.0×10^7	35.5
2.7×10^7	33.5	1.7×10^7	33.5
1.5×10^7	31.4	1.9×10^7	31.4
2.1×10^7	29.4	1.7×10^7	29.4
1.6×10^7	27.3	1.3×10^7	27.3
2.1×10^7	25.2	1.5×10^7	25.2
2.4×10^7	23.1	1.1×10^7	23.1
1.6×10^7	21.1	1.1×10^7	21.1
1.6×10^7	19.0	8.5×10^6	19.0
7.5×10^6	17.0	3.2×10^6	17.0
3.0×10^6	15.0	4×10^5	15.0

30 min treatment		35 min treatment	
Converted average colony count/ml	incubation temperature	Converted average colony count/ml	incubation temperature
<5	54.0	<5	54.0
<5	52.1	<5	52.1
<50	50.2	<5	50.2
215	48.4	650	48.4
2×10^3	46.5	3.2×10^4	46.5
4.9×10^5	44.7	1.4×10^5	44.7
4.6×10^5	42.8	2.9×10^5	42.8
8.2×10^6	41.0	4.8×10^5	41.0
1.1×10^7	39.1	6.2×10^5	39.1
1.3×10^7	37.1	7.2×10^5	37.1
1.4×10^7	35.3	7.7×10^5	35.3
1.3×10^7	33.4	8.0×10^5	33.4
1.6×10^7	31.5	8.6×10^5	31.5
1.5×10^7	29.8	7.7×10^5	29.8
1.8×10^7	27.9	7.7×10^5	27.9
1.7×10^7	26.0	5.2×10^5	26.0
1.1×10^7	24.1	5.3×10^5	24.1
1.2×10^7	22.2	5.1×10^5	22.2
1.1×10^7	20.3	4.1×10^5	20.3
7.7×10^6	18.5	4.9×10^5	18.5
2.3×10^6	16.75	5.5×10^5	16.75

40 min treatment	
Converted average colony count/ml	incubation temperature
<5	54.0
<5	52.1
<5	50.2
250	48.4
8.1×10^3	46.5
2.1×10^4	44.7
4.1×10^4	42.8
6.6×10^4	41.0
1.0×10^5	39.1
1.2×10^5	37.1
1.4×10^5	35.3
1.3×10^5	33.4
1.5×10^5	31.5
1.3×10^5	29.8
1.2×10^5	27.9
1.4×10^5	26.0
1.0×10^5	24.1
6.6×10^4	22.2
6.7×10^4	20.3
5.5×10^4	18.5
3.8×10^4	16.75

45 min treatment	
Converted average colony count/ml	incubation temperature
<5	55
<5	53
<5	51
150	49
400	47
3.1×10^3	45.5
6.0×10^3	43.5
1.3×10^4	41.5
2.1×10^4	40
2.3×10^4	38
3.0×10^4	36
3.6×10^4	34
3.6×10^4	32
3.4×10^4	30
3.8×10^4	28.5
3.2×10^4	26.5
2.8×10^4	24.5
2.7×10^4	22.5
2.8×10^4	21
1.3×10^4	19
7.5×10^3	17

50 min treatment		55 min treatment	
Converted average colony count/ml	incubation temperature	Converted average colony count/ml	incubation temperature
<5	54.0	<5	55
<5	52.1	<5	53
<5	50.2	<5	51
<5	48.5	<5	49
180	46.8	10	47
350	44.8	35	45.5
3.2×10^3	42.9	300	43.5
6.2×10^3	41.1	650	41.5
1.0×10^4	39.2	1.4×10^3	40
1.2×10^4	37.1	1.7×10^3	38
1.6×10^4	35.2	2.2×10^3	36
1.7×10^4	33.6	2.3×10^3	34
1.8×10^4	31.9	2.1×10^3	32
1.9×10^4	30.0	2.4×10^3	30
2.0×10^4	28.0	2.9×10^3	28.5
1.9×10^4	26.2	2.1×10^3	26.5
1.9×10^4	24.4	1.9×10^3	24.5
1.5×10^4	22.6	2.0×10^3	22.5
1.5×10^4	20.6	1.1×10^3	21
1.2×10^4	19.0	1.0×10^3	19
5×10^3	17.0	4.4×10^2	17

60 min treatment		65 min treatment	
Converted average colony count/ml	incubation temperature	Converted average colony count/ml	incubation temperature
<5	54.0	<5	54.0
<5	52.1	<5	52.1
<5	50.2	<5	50.2
<5	48.5	<5	48.5
5	46.8	5	46.8
75	44.8	70	44.8
2.9×10^2	42.9	185	42.9
4.6×10^2	41.1	280	41.1
8.9×10^2	39.2	505	39.2
1.1×10^3	37.1	740	37.1
1.5×10^3	35.2	985	35.2
1.7×10^3	33.6	1.1×10^3	33.6
1.9×10^3	31.9	1.5×10^3	31.9
1.4×10^3	30.0	1.4×10^3	30.0
1.2×10^3	28.0	1.3×10^3	28.0
1.6×10^3	26.2	1.5×10^3	26.2
1.6×10^3	24.4	1.3×10^3	24.4
1.5×10^3	22.6	1.3×10^3	22.6
1.3×10^3	20.6	1.0×10^3	20.6
9.6×10^2	19.0	6.8×10^2	19.0
-	17.0	3.3×10^2	17.0

70 min treatment	
Converted average colony count/ml	Incubation temperature
<5	54.0
<5	52.1
<5	50.2
<5	48.5
<5	46.8
10	44.8
15	42.9
55	41.1
85	39.2
140	37.1
125	35.2
95	33.6
125	31.9
205	30.0
185	28.0
190	26.2
230	24.4
240	22.6
165	20.6
105	19.0
25	17.0

2. The effect of incubation temperature on the recovery in Brain Heart Infusion Agar of *B. subtilis* 8057 spores after treatment at 95° for different times (see Fig. 8).

0 treatment		10 min treatment	
Converted average colony count/ml	Incubation temperature	Converted average colony count/ml	Incubation temperature
1.1×10^5	54	1.1×10^5	53.5
1.2×10^7	52	1.5×10^6	52
1.8×10^7	50	8.6×10^6	50
1.5×10^7	48.5	1.3×10^7	48
1.7×10^7	46.5	1.2×10^7	46
1.8×10^7	44.5	1.2×10^7	44
1.9×10^7	43	1.5×10^7	42
1.7×10^7	41	1.2×10^7	40.5
1.9×10^7	39	1.4×10^7	38.5
2.0×10^7	37.5	1.5×10^7	36.5
1.7×10^7	35.5	1.3×10^7	35
1.8×10^7	33.5	1.3×10^7	33
1.6×10^7	32	1.2×10^7	31
1.8×10^7	30	1.2×10^7	29
1.9×10^7	28	1.3×10^7	27.5
1.8×10^7	26	1.3×10^7	25.5
2.1×10^7	24.5	1.3×10^7	23.5
2.1×10^7	23	1.4×10^7	21.5
2.2×10^7	21	1.4×10^7	20
2.2×10^7	19	1.3×10^7	18
2.1×10^7	17	1.2×10^7	16

20 min treatment	
Converted average colony count/ml	Incubation temperature
<50	53.5
<50	51.5
2.9×10^4	49.5
5.6×10^5	48
1.8×10^6	46
2.7×10^6	44
3.1×10^6	42
4.0×10^6	40.5
4.1×10^6	38.5
4.2×10^6	36.5
4.0×10^6	35
3.6×10^6	33
4.2×10^6	31
4.0×10^6	29
4.0×10^6	27.5
3.4×10^6	25.5
3.2×10^6	23.5
3.9×10^6	21.5
3.3×10^6	20
2.8×10^6	18
1.3×10^6	16

30 min treatment	
Converted average colony count/ml	Incubation temperature
<50	53.5
<50	51.5
1.1×10^2	49.5
3.5×10^3	48
8.3×10^4	46
1.8×10^5	44
3.2×10^5	42
4.7×10^5	40.5
6.1×10^5	38.5
6.9×10^5	36.5
7.1×10^5	35
7.1×10^5	33
8.2×10^5	31
7.7×10^5	29.5
8.5×10^5	27.5
9.3×10^5	25.5
9.5×10^5	23.5
7.3×10^5	21.5
7.2×10^5	20
5.4×10^5	18
1.5×10^5	16

40 min treatment		50 min treatment	
Converted average colony count/ml	Incubation temperature	Converted average colony count/ml	Incubation temperature
<5	53.5	<5	54
<5	51.5	<5	52
<5	49.5	<5	50
4.5×10^2	48	45	48
6.3×10^3	46	6.8×10^2	46.5
1.3×10^4	44	1.4×10^3	44.5
2.9×10^4	42	3.0×10^3	42.5
4.6×10^4	40.5	4.9×10^3	41
7.5×10^4	38.5	8.1×10^3	39
1.0×10^5	36.5	1.2×10^4	37
1.1×10^5	35	1.6×10^4	35
1.2×10^5	33	1.4×10^4	33
1.3×10^5	31	1.7×10^4	31
1.2×10^5	29	1.4×10^4	29.5
1.4×10^5	27.5	1.4×10^4	27.5
1.2×10^5	25.5	1.6×10^4	25.5
1.3×10^5	23.5	1.6×10^4	23.5
1.2×10^5	21.5	1.1×10^4	21.5
6.4×10^4	20	9.9×10^3	20
7.1×10^4	18	4.2×10^3	18
2.0×10^4	16	1.3×10^3	16

60 min treatment		70 min treatment	
Converted average colony count/ml	Incubation temperature	Converted average colony count/ml	Incubation temperature
<5	54	<5	54
<5	52	<5	52
<5	50	<5	50
<5	47.5	<5	47.5
38	45.5	<5	45.5
80	43.5	15	43.5
6×10^2	41.5	25	41.5
3.2×10^2	40	50	40
5.2×10^2	38	59	38
1.6×10^3	36	140	36
1.1×10^3	34	190	34
1.7×10^3	32.5	160	32.5
1.4×10^3	30.5	230	30.5
1.8×10^3	28.5	190	28.5
2.4×10^5	27	210	27
1.6×10^3	25	270	25
2.1×10^3	23	200	23
8.1×10^2	21	180	21
9.0×10^2	18.5	130	18.5
6.1×10^2	17.5	95	17.5
2.1×10^2	15.5	25	15.5

3. The effect of incubation temperature on the recovery in Plate Count Agar of *B. subtilis* spores after treatment at 105° for different times (see Fig. 9).

0 treatment		4 min treatment	
Converted average colony count/ml	Incubation temperature	Converted average colony count/ml	Incubation temperature
1.5×10^8	54	$<5 \times 10^4$	53
1.2×10^8	52	$<5 \times 10^4$	51
1.4×10^8	50	7.0×10^5	49.5
1.3×10^8	48	1.3×10^7	48
1.3×10^8	46	4.7×10^7	46
1.5×10^8	44	3.7×10^7	44
1.9×10^8	42.5	6.7×10^7	42
1.5×10^8	41	6.7×10^7	40.5
1.8×10^8	39	7.9×10^7	39
1.7×10^8	37	7.7×10^7	37
1.4×10^8	35	9.7×10^7	35
1.2×10^8	33	9.6×10^7	33.5
1.7×10^8	31.5	1.0×10^8	31.5
1.7×10^8	29.5	1.0×10^8	30
1.5×10^8	28	9.9×10^8	28
1.6×10^8	26	1.0×10^8	26
1.4×10^8	24	8.9×10^7	24.5
1.8×10^8	22	7.9×10^7	22.5
1.6×10^8	20	8.7×10^7	21
1.5×10^8	18	8.1×10^7	19
1.6×10^8	16.5	8.0×10^7	17

6 min treatment		8 min treatment	
Converted average colony count/ml	Incubation temperature	Converted average colony count/ml	Incubation temperature
$<5 \times 10^4$	53.5	<50	53.5
$<5 \times 10^4$	51.5	<50	51.5
$<5 \times 10^4$	49.5	<50	49.5
$<5 \times 10^4$	48	<50	48
2.7×10^6	46	<50	46
8.3×10^6	44	3×10^1	44.5
1.5×10^7	42.5	3×10^1	42.5
3.4×10^7	40.5	2.8×10^2	40.5
4.4×10^7	39	8.5×10^3	39
3.8×10^7	37	3.0×10^4	37
4.7×10^7	35.5	6.5×10^4	35
4.6×10^7	33.5	8.2×10^4	33.5
5.6×10^7	32	8.8×10^4	31.5
4.6×10^7	30	7.8×10^4	30
4.6×10^7	28	5.6×10^4	28
3.4×10^7	26.5	7.5×10^4	26
3.3×10^7	25	4.5×10^4	24.5
3.1×10^7	23	3.2×10^4	22.5
2.0×10^7	19	1.2×10^4	19
1.5×10^7	17.5	6.8×10^3	17

10 min treatment	
Converted average colony count/ml	Incubation Temperature
<50	54
<50	52
<50	50
<50	48
<50	46
<50	44
<50	42
<50	40
1.5×10^2	38
6.5×10^3	36
8.5×10^3	34
1.2×10^4	32.5
1.6×10^4	30.5
1.3×10^4	28.5
1.3×10^4	26.5
6.5×10^3	24.5
7.5×10^3	23
5.5×10^3	21
1.5×10^3	19
<50	-
<50	-

4. The germination of _B. subtilis_ 8057 spores at 20° after treatment at 95° for different times
(See Fig. 12)

Time of Incubation	average colony count/ml 0 treatment	30 min treatment	60 min treatment
0	1.8×10^6	2.3×10^5	7.1×10^2
5 min	7.6×10^6	2.5×10^5	6.4×10^2
10 min	7.7×10^5	2.1×10^5	5.9×10^2
15 min	6.9×10^5	1.9×10^5	7.4×10^2
20 min	6.6×10^5	1.7×10^5	5.3×10^2
30 min	6.1×10^5	1.3×10^5	4.5×10^2
45 min	6.4×10^5	8.0×10^4	4.1×10^2
60 min	4.5×10^5	4.2×10^4	3.5×10^2
90 min	7.2×10^5	1.8×10^4	2.3×10^2
2 h	3.3×10^5	1.5×10^4	1.4×10^2
3 h	1.7×10^5	7.3×10^3	1.5×10^2
4 h	9.5×10^4	3.3×10^3	80
6 h	3.8×10^4	1.2×10^3	70
8 h	1.5×10^4	5.0×10^2	55
10 h	7.2×10^3	1.6×10^2	<10
12 h	7.7×10^3	95	<10
14 h	9.9×10^3	<10	<10
16 h	2.1×10^3	<10	<10
18 h	5.3×10^4	<10	<10
20 h	5.8×10^3	<10	<10
22 h	6.7×10^2	<10	<10
24 h	4.8×10^2	<10	<10

5. The germination of *B. subtilis* 8057 spores at 30° after treatment at 95° for different times
(See Fig. 13)

	average colony count/ml		
Time of Incubation	0 treatment	30 min treatment	60 min treatment
0 min	1.6×10^6	9.6×10^4	170
5 min	8.4×10^5	5.7×10^4	130
10 min	4.7×10^5	5.6×10^4	140
15 min	1.1×10^5	4.8×10^4	30
20 min	5.9×10^4	3.5×10^4	30
30 min	1.1×10^3	3.9×10^4	290
45 min	1.4×10^2	2.3×10^4	130
60 min	730	2.1×10^4	110
90 min	870	1.2×10^4	75
2 h	230	5.1×10^3	70
3 h	240	1.6×10^3	40
4 h	18.5×10^2	600	20
6 h	1.1×10^5	100	10
8 h	9.4×10^5	190	10
10 h	2×10^4	360	-
12 h	3.5×10^5	3.1×10^4	-

6. The effect of incubation temperature on the outgrowth of *B. subtilis* 8057 spores after treatment at 95° for different times and incubation at 37° for 30 min (see Fig. 20).

0 treatment		20 min treatment	
Converted average colony count/ml	Incubation temperature	Converted average colony count/ml	Incubation temperature
2.7×10^8	50	1.7×10^8	50
1.1×10^9	46.5	5.3×10^8	46.5
1.0×10^9	43	8.3×10^8	43
1.2×10^9	39.5	1.2×10^9	39.5
1.6×10^9	36	1.5×10^9	36
1.8×10^9	32	1.5×10^9	32
1.9×10^9	28.5	1.3×10^9	28.5
2.3×10^9	25	1.5×10^9	25
2×10^9	21.5	1.0×10^9	21.5
1.5×10^9	18	1.0×10^9	18
8.8×10^8	16	7.3×10^8	16

40 min treatment	
Converted average colony count/ml	Incubation temperature
<50	52
2.9×10^5	48
2.1×10^7	42.5
4.5×10^7	39
6.3×10^7	35
7.4×10^7	31.5
7.0×10^7	28
8.0×10^7	24
7.0×10^7	20.5
3.7×10^7	17
1.5×10^7	15

60 min treatment	
Converted average colony count/ml	Incubation temperature
2.4×10^4	51
1.9×10^4	47
3.6×10^4	43.5
4.4×10^4	40
5.4×10^4	36.5
8.2×10^4	33
7.1×10^4	29.5
4.3×10^4	26
4.8×10^4	22
4.5×10^4	18.5

7. The effect of incubation temperature on the outgrowth of B. subtilis 8057 spores after treatment at 95° for 60 min and incubation at 37° for 60 min (see Fig. 21).

Incubation Temperature	Converted average colony count/ml
52	<50
48.5	6.4×10^5
46.5	3.3×10^6
43	1.3×10^8
39.5	4.3×10^8
36	5.8×10^8
32	7.0×10^8
28.5	9.2×10^8
25	8.6×10^8
21.5	6.0×10^8
18	1.7×10^8